UNIVERSITY OF LONDON THESIS

Degree phD Year 2007 Name of Author ANASTASIOS CHANALARIS

TITLE

EFFECTS OF THE UROCORTIN FAMILY OF PEPTIDES ON CARDIAC NEONATAL MYOCYTES.

ANTIAPOPTOTIC AND HYPERTROPHIC EFFECTS.

SUBMITTED BY: CHANALARIS ANASTASIOS

REGISTERED AT THE INSTITIUTE OF CHILD HEALTH, UCL

SUBMITTED FOR THE DEGREE OF PHD

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To Rachel Davidge and my family, for their love, faith in my abilities and ceaseless support.

"The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error."

Bertolt Brecht, *The life of Galileo* (sc.9)

Nothing in biology makes sense except in the light of evolution.

Theodosius Dobzhansky

ABSTRACT

Urocortin 1 (UCN) and its homologues Urocortin 2 (UCN2/SRP) and urocortin 3 (UCN3/SCP) belong to the corticotropin releasing factor (CRF) family of peptides. CRF peptides have been shown to affect cardiovascular physiology.

The effects of the peptides on cardioprotection after hypoxia/reoxygenation injury and on hypertrophy were examined. As SCP and SRP have a higher affinity for the CRF receptor 2β, the only CRF receptor expressed in the rat heart, compared to UCN that additionally binds to the CRF receptor 1, the hypothesis was posed that the actions of SCP and SRP on cardiomyocytes will be more pronounced and more specific than UCN. As the pathways that are downstream of CRF receptors are not fully characterised, a possible mechanism of action is examined.

The protective effect of the three peptides was compared and it was shown that all three are protective. Moreover, SCP and SRP are induced by hypoxia/reoxygenation injury.

Examining the hypertrophic effects of the peptides it was concluded that all three exhibited such properties with SCP being the most potent. Furthermore, it is shown that UCN homologues require activation of MAPK p42/44 and PKB/Akt for their cardioprotective effects, but only PKB/Akt for hypertrophy.

The involvement of two other important signalling molecules, $iPLA_2$ and protein kinase C_ε (PKC_ε) was also examined. $iPLA_2$ protein levels are down-regulated by UCN peptides, attenuating the effects of the enzyme after hypoxia/reoxygenation. PKC_ε protein levels are induced by UCN peptides indicating that PKC_ε is involved in the action of the peptides. Finally, UCN peptides upregulate the Sensitive to Apoptosis Gene (SAG) and

the K_{ATP} channel, Kir 6.1 and these are two of the downstream effectors of cardioprotection. In addition, SAG appears to be a cardioprotective molecule that interacts with caspase-3.

In conclusion, UCN peptides generate a multifaceted effect on cardiomyocyte physiology and we provide information on new mechanisms of action by the peptides. In addition, we illustrate that SCP is the most potent peptide of the three and provide additional evidence on the notion that C-terminal amidation of the CRF peptides is a requirement for their biological effect.

TABLE OF CONTENTS

ABBREVIATIONS

5-HD: 5-hydroxy-decanoate
ACTH: Adrenocorticotropic Hormone
ANOVA: Analysis of Variance
ANP/ANF: Atrial natriuretic Peptide/Factor
Apaf-1: apoptosis promoting factor-1
APC: Anaphase Promoting Complex
Apg: Autophagy-defective gene
ARC: Apoptosis Repressor with CARD
Arg: Arginine
Asp: Aspartic acid
ATP: Adenosine triphospate nucleotide
Bcl-2: B-cell lymphoma protein -2
BEL: Bromo enol lactone
BMK: Big MAP kinase
BNP/BNF: Brain natriuretic peptide/factor
CAD: Caspase Activated DNase
cAMP: cyclic adenosine monophosphate nucleotide
CBC: Elongin C-Elongin B Complex
CHD: Coronary Heart Desease
CRF/CRH: Corticotropin releasing Factor/Peptide
CRFR/CRHR: Corticotropin Releasing Factor/Peptide Receptor
Cul: Culin
Cys: Cysteine
DAG: Diacyl glycerol
DNA: Dysdeoxynucleotidic acid
DUP: Deubiquitinating protein
ECG: Electrocardiogram
ERK: Extracellular regulated kinase
ET-1: Endothelin-1
FACS: Flow assisted cell sorting
FADD: Fas associated death domain
Gly: Glycine
gp130: glycoprotein 130
GPCR: G-protein coupled receptor
HECT: Homologous to the E6-AP carboxy terminus
HIF: Hypoxia induced factor
His: Histidine
HPV: Human Papiloma Virus
Hsp: Heat Shock Protein
IAP: Inhibitor of Apoptosis
IBR region: In Between RING region
IGFR-1: Insulin Growth Factor Receptor-1
IκB: Inhibitor of NFκB

JAK: Janus kinase
JNK: c-Jun N-terminal Kinase
K_{ATP} channel: ATP regulated potassium channel
Kip: Kinase inhibitor protein
Kir: Inwardly rectifying potassium channel
LPA: Lysophosphatidic Acid
LPC/Lyso PC: Lysophosphatidyl choline
MAPK: Mitogen Activated Protein Kinase
MEK: Mitogen activated protein Kinase Kinase
NBD: N Binding Protein
NFκB: nuclear factor κB
PARP: Poly-ADP Ribose Polymerase
PCR: Polymerase Chain Reaction
PDK: 3'-Phospoinositide dependent Kinase
PI-3K: Phospho- Inosytol 3 Kinase
PKB/Akt: Protein kinase B
PKC: Protein kinase C
PKN: Protein Kinase N
PLA: Phospholipase
PMA: Phorbol 12-myristate -13-acetate
PRK: Parkin
PRT1: Proteolysis 1
PS: phosphatidyl Serine
RACK: membrane-bound receptor for activated protein kinase C proteins
RING: Really interesting gene
RNA:Ribonucleic acid
mRNA: messenger Ribonucleic Acid
ROC: regulator of Cullin
ROS: Reactive Oxygen Species
RUB1/Nedd8: Related to Ubiquitin protein 1
SAG: Sensitive to apoptosis Gene
SAPK: Stress associated protein kinase
SCF: Skp1/Cul/F-box protein complex
SCP: stresscopin
SEK-1/MKK4/JKK1: Mitogen activated Kinase Kinase 4
SEM: Standard Error of mean
Ser: Serine
Skp1: Suppressor of Kinetochore 1
SOCS: Suppressors of Cytokine Signalling
SRP: Stresscopin related protein
SUMP: Small Ubiquitin-like Modifier Protein
SUR: sulfonylurea receptor
SVG: sauvagine
Thr: Threonine
TNF-α: Tumor necrosis factor
TRADD: TNF receptor associated death domain

TUNEL: Tdt-mediated dUTP-biotin nick end labeling
Tyr: Tyrosine
UBC: Ubiquitin Cojugating Protein
UCN: Urocortin
UCPR: Ubiquitin conjugating E2 related protein
UDP: Uridine Diphosphonucleotide
URO: urotensin
UV: ultraviolet
VEGF: Vascular endothelial growth factor
VHL: Von Hippel-Lindau
β-TrCP: beta-Transducin repeat-Containing Protein

CHAPTER I

1. GENERAL INTRODUCTION

1.1 CARDIOVASCULAR DISEASES

The heart is one of the major organs of the body. Its function is to pump blood around the body. It is mainly comprised of differentiated muscle cells, the cardiomyocytes. Cardiomyocytes, being terminally differentiated cells, rarely divide. However, recent reports show that cell division might be occurring among cardiomyocytes in failing hearts (Kajstura et al., 1998). Nevertheless, the rate of division is too small to be able to make up for the loss of cells in an infarct area. It is not surprising therefore that Cardiovascular Diseases are the major killer worldwide, accounting for approximately 12 million deaths annually (Gill et al., 2002).

Coronary heart diseases are caused by the obstruction of the blood flow in the coronary arteries, leading to minimised oxygenation and nutrient supply of the cardiomyocytes. This insufficient blood flow in the coronary arteries is called ischaemia. As a result, cardiomyocyte physiology becomes compromised and may lead to cell death and ultimately heart failure.

Restoration of blood flow after an ischaemic episode is called reperfusion and although it is required for the survival of the cardiac tissue, the reintroduction of oxygen in an ischaemic cell, is the main cause for the cellular injury that is described by the term reperfusion injury.

Cardiac hypertrophy is the cellular response to an increase in biomechanical stress, either extrinsic as in arterial hypertension or valvular heart disease, or intrinsic as in familial hypertrophic cardiomyopathy (Braunwald et al., 2002; Marian, 2002; Wilkins and Molkentin, 2002; Lips et al., 2003). Cardiac hypertrophy is a compensatory mechanism striving to normalise the increase in wall tension. Hypertrophy in response to pathologic stimuli has been considered an adaptive response, required in order to sustain cardiac output during stress (Braunwald et al., 2002; Marian, 2002; Wilkins and Molkentin, 2002; Lips et al., 2003). However, prolonged hypertrophy is associated with a significant increase in the risk for sudden death or progression to heart failure, independent of the underlying cause of hypertrophy, suggesting that the hypertrophic response is not entirely beneficial (Braunwald et al., 2002; Marian, 2002; Wilkins and Molkentin, 2002; Lips et al., 2003).

Current clinical methodologies do not allow us to measure the impact of mortality caused by hypertrophic heart failure. However, it is well documented that hypertrophic heart failure has a considerable contribution to the mortality of patients (Braunwald et al., 2002; Lips et al., 2003).

Coronary heart disease is estimated to cost the UK economy a total of £7,055 million in 1999 alone. This represents a cost that is higher than that for any other single disease for which a comparable analysis has been carried out (Petersen et al., 2003).

Regardless of the enormous cost of the disease in the UK economy, the death rate from CHD in the UK is amongst the highest in the world (Petersen et al., 2003). Clearly, current strategies for disease prevention and therapy are not entirely adequate, even if the rate of deaths from CHD has been falling in the UK. Deaths from cardiovascular diseases constituted 40% of all causes of death in the UK in 2001 (Petersen et al, 2003). Coronary Heart disease is accountable for half of them and is the single biggest killer in the UK alone.

1.2 CELL DEATH

The mode of cell death of the cardiomyocytes in ischaemia-reperfusion injury involves both necrosis and apoptosis (Kajstura et al., 1996; Grubel et al., 1998; Takahashi and Ashraf, 2000 and Gill et al., 2002). The extent to which each form of cell death contributes to the formation of the infarct is not yet clear. Although both necrosis and apoptosis result in the death of the cell, they differ in several morphological and cellular regulatory features.

1.2.1 Necrosis

Necrosis is a rapid and irreversible process that occurs when cells are severely damaged (Table 1.1). Necrosis is characterised by mitochondrial and cytoplasmic swelling, membrane rupture and cell lysis. The release of the cellular contents into the extracellular milieu causes further damage to neighbouring cells, leading to secondary inflammation. Phagocytosis of the remnants is delayed until accumulation of inflammatory cells. The resulting lesions contain groups of necrotic cells and inflammatory leucocytes (Saraste and Pulkki 2000; Yaoita et al., 2000; Gill et al., 2002 and Krijnen et al., 2002).

1.2.1.1 Necrosis in myocardial ischaemia

Myocardial necrosis has been well documented in animal studies, and it has been shown that it develops rapidly after ischaemia (Zhao and Vinten-Johansen, 2000). Work done in rat hearts where the left coronary artery was occluded for 10-30 minutes and reperfused for 120 minutes, showed that myocytes undergo necrosis after 20 minutes of occlusion and 26.6% percent of the cells in the infarct area were found to be necrotic in this study (Takahashi and Asraf, 2000). A similar study in rats has also shown that necrotic cell death is observed in cardiomyocytes in heart that was subjected to coronary artery occlusion for 20 minutes to 7 days. The results show that necrotic cells do exist in the infarct area and that the amount of necrotic cells was increasing with increasing duration of occlusion and peaked after 1 day. However, the study showed that apoptosis was the main form of cell death of the cardiomyocytes in the infarct area (Grubel et al., 1998).

Similarly, in a chronic canine model where the heart was subjected to 6-72 hours of reperfusion following 1hour of ischaemia, the necrotic area increased with increasing time of reperfusion and peaked after 24 hours of reperfusion (Zhao et al., 2000).

1.2.2 Apoptosis

Apoptosis is used as a contrasting term to necrosis. Apoptosis is a highly organised and energy dependent form of death distinguished in its later stages, by the activation of a specific group of proteases, the caspases. Apoptosis is characterised by membrane blebbing, cell shrinkage and formation of apoptotic bodies, chromatin condensation, DNA fragmentation and exposure of phosphatidyl serine on the outer leaf of the plasma membrane prior to membrane disintegration, finally leading to cell death. There is no resulting inflammation and the apoptotic cells are phagocytosed (Saraste and Pulkki 2000; Yaoita et al., 2000; Gill et al., 2002 and Krijnen et al., 2002). Table 1.1 shows the main differences between apoptosis and necrosis.

1.2.2.1 Apoptosis in ischaemia and hypertrophy

Apoptotic cell death is an important process in the pathology of heart disease (Fliss and Gattinger, 1996 and Narula et al., 1996). Myocyte apoptosis has been demonstrated in humans suffering from myocardial infarction as well as in rabbit, rat and mouse models of cardiac ischaemia or ischaemia followed by reperfusion (Gottlieb et al., 1994; Cheng et al., 1996; Tomei and Umansky, 2001; Stephanou et al., 2001; Scarabelli et al., 2002a). In order to prevent extensive death after an ischaemic episode, the myocardium needs to be reperfused to restore the

TABLE 1.1

Apoptosis vs. necrosis (Adapted from Yaoita et al., 2000)

	Apoptosis	**Necrosis**
Plasma membrane	Intact until late in the process	Destroyed early
Morphological features	• Chromatin condensation • Nuclear fragmentation • No mitochondrial swelling until late • Cell shrinkage	• Swelling of entire cytoplasm • Mitochondrial and organelle swelling
Biochemical features	• DNA fragmentation by endonucleases • Protein degradation by caspases • Surface exposure of phosphatidyl serine	• Non-specific DNA degradation • Non specific protein degradation
Fate	Heterophagic elimination and little inflammation	Cell lysis and secondary inflammation
Biological meaning	Physiological and pathological, tightly regulated	Accidental and unregulated

nutrient and oxygen supply. However, reperfusion has been shown to result in augmentation of the damage in the myocardium. Two hypotheses have been brought forward to explain the injury after reperfusion (Ashraf, 1997):

- The Calcium hypothesis,
- and the free radical hypothesis.

The calcium hypothesis suggests that ischaemia induces a defect in the cardiomyocyte's ability to regulate calcium and upon reperfusion the cell accumulates toxic levels of calcium. Calcium overload can be detrimental to the fate of the cell and can cause cell death. Upon reoxygenation mitochondria excessively accumulate calcium, which compromises ATP synthesis (Ashraf, 1997).

The calcium influx occurs upon reperfusion through the damaged sarcolemma (Ashraf, 1997). Calcium influx activates Ca-dependent cysteine proteases, calpains, which can activate pro-caspase-12 (Yaoita et al., 2000). Calpains can also cleave Bax, promoting its antiapoptotic effects (Gill et al., 2002).

The free radical hypothesis of reperfusion injury is based on the rationale that partially reduced forms of molecular oxygen including the superoxide anion ($\cdot O^-_2$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($\cdot OH$) are produced at the time of reperfusion or introduction of molecular oxygen O_2. Free radical scavengers, such as superoxide dismutase (SOD) and catalase, can reduce the infarct size in regional ischaemia model and support the free radical

hypothesis (Asraf, 1997). The free radicals are highly reactive and can readily oxidise lipids and proteins causing damage to a number of enzymes and cell membranes. Asraf proposes that free radicals might be inducing membrane defects which promote calcium entry, thus unifying both these theories (Asraf, 1997).

H_2O_2 treatment has been shown to induce cytochrome C release, activation of caspase-3 and poly-ADP-ribose polymerase (PARP) cleavage (von Harsdorff et al., 1999a). Also, free radicals can directly induce mitochondrial and DNA damage and p53 activation (Ashraf, 1997).

Activation of caspases occurs in cardiomyocytes through two main pathways. The first pathway is the death receptor pathway (Fig. 1.1).

Both of the death receptors Fas and TNF-α Receptor are involved in ischaemia reperfusion injury in the heart. Fas has been shown to be involved in cell death following myocardial ischaemia (Jeremias et al., 2000). TNF-α can also induce apoptosis in cardiomyocytes (Krown et al., 1996). Activation of the Death receptors lead to a cascade of events that lead to the activation of a specific caspase, caspase-8 (Fig. 1.1).

The second major apoptotic pathway involves the mitochondrion and is therefore called the mitochondrial pathway. Mitochondria are one of the most important

organelles in the heart. They are not only producing essential energy for the cardiomyocytes, but can also regulate its survival. The Bcl-2 family of apoptosis regulatory proteins are acting on the mitochondrion and free radicals can be produced in the mitochondrion.

Cytochrome C release from the mitochondrion is one of the main mechanisms of apoptosis in cardiomyocytes. Cytochrome C release results in formation of the apoptosome, a protein complex formed by Apaf-1 (apoptosis promoting factor-1), cytochrome C and procaspase-9, in the presence of dATP. Formation of the apoptosome results in cleavage of procaspase-9 to active caspase-9 and activation of caspase-3 (Fig. 1.1).

Caspase-3, or executioner caspase, so called because it is activated by both the major apoptotic pathways and to differentiate it from caspases 8 and 9 (the activating caspases) that activate it, cleaves a number of proteins in the cell and activates caspase-activated DNAse (CAD) that is needed for DNA fragmentation and is one of the last steps in a point of no return for apoptosis (Fig. 1.1).

There is a great need to characterise the pathways that regulate apoptosis in the heart, in order to establish new therapeutic strategies in failing hearts. There is considerable knowledge of the pathways that lead to activation of caspases from extracellular signals and from mitochondrial damage. There is also considerable interest in genes that are characterised as anti-apoptotic such as the inhibitors of

apoptosis (IAPs) (Fig. 1.1), which inhibit the onset of apoptotic cascades by interacting with caspases and preventing their activation (Goyal, 2001). However, there is so far little knowledge on how these pathways are regulated and on ways that we could exploit them in order to minimise the amount of cell death that occurs in the heart after hypoxia/reoxygenation.

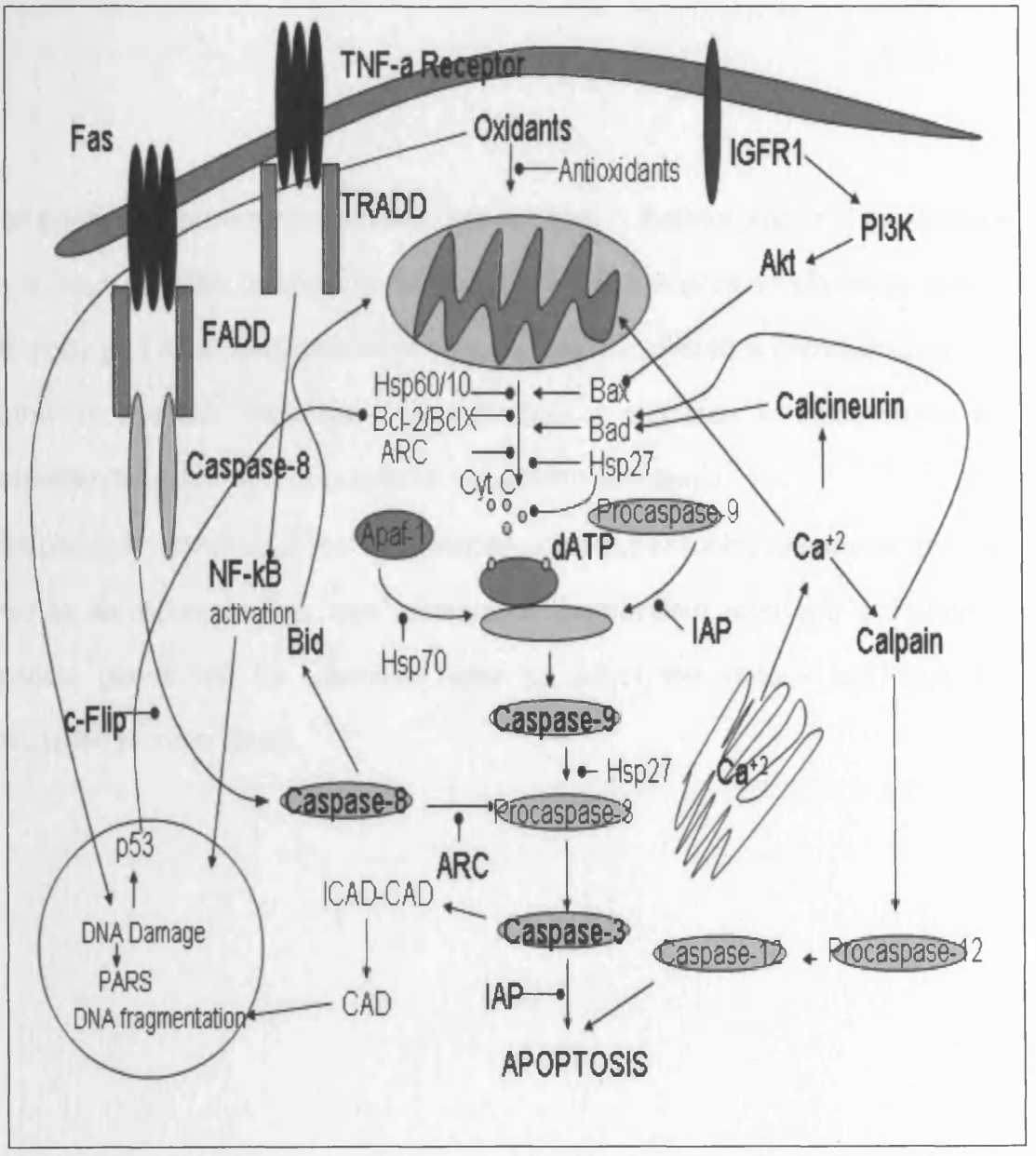


Figure 1.1. Schematic overview of major apoptotic pathways involved in ischaemic cardiomyocytes. Arrows indicate activation, whereas clubs indicate inhibition.

Cell death in hypertrophic diseases only appears in the late stages of the disease as a result of heart failure. The heart is unable to pump blood efficiently around the body and as a result the blood supply to the heart itself is compromised. The pathways that are responsible for activation of apoptosis in these cases are generally the same as in the case of myocardial ischaemia.

The pathways involved in the early stages of the hypertrophic responses and that lead to an increased cell size, increase in protein:DNA ratio and activation of specific genes will be examined later on when the various pathways are discussed in more detail.

1.3 Signalling Pathways in cardiomyocytes that are involved in Ischaemia/reperfusion and hypertrophy

Protein kinases constitute the main mechanism of signal transduction in the cells and have been involved in every aspect of cell life regulating cell homeostasis and driving the cell to grow, divide, differentiate or die (Johnson and Lapadat, 2002).

Protein kinases are enzymes that covalently attach phosphate to the hydroxyl group of serine, threonine or tyrosine of specific proteins inside cells. Such phosphorylation of proteins can control their enzymatic activity, their interaction with proteins and other molecules, their location inside the cell, and their propensity for degradation from proteases. The kinases are divided into two main groups, according to the hydroxyl group of the amino acid that they phosphorylate. The two groups are the Ser/Thr kinases and the Tyr kinases (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001; Johnson and Lapadat, 2002).

In cardiomyocytes, protein kinases can regulate contraction, ion transport, fuel metabolism, growth and survival or death (Sugden and Bogoyevitch, 1995). We will examine the most relevant kinases that are involved in ischaemia/reperfusion and in hypertrophy in turn.

1.3.1 Mitogen Activated Protein Kinases (MAPK)

MAPKs are Ser/Thr kinases that regulate the expression of specific sets of genes and can modulate specific responses to extracellular stimuli (Johnson and Lapadat, 2002).

They are part of signalling cascades consisting of three levels, a MAPK kinase kinase, which activates a MAPK kinase, which finally activates the MAPK (Fig. 1.3) (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001; Johnson and Lapadat, 2002). MAPKs compose a family of protein kinases whose function and regulation have been conserved from unicellular organisms to humans (Johnson and Lapadat, 2002). They are phosphorylated after activation of G-protein coupled receptors, and receptor protein tyrosine kinases (Fig. 1.3) (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001; Johnson and Lapadat, 2002).

MAPKs are divided into three groups. In the heart the three groups are comprised of the:

- Extracellular signalling regulated kinases (ERK -1 and -2, or p42 and p44 MAPK),
- BMK1/ERK 5 or Big MAPK.
- Jun N-terminal kinases 1 and 2 (JNK 1 and 2)
- P38 MAPKs (p38α, p38β, p38-2, SAPK-3/p38γ and SAPK-4/p38δ).

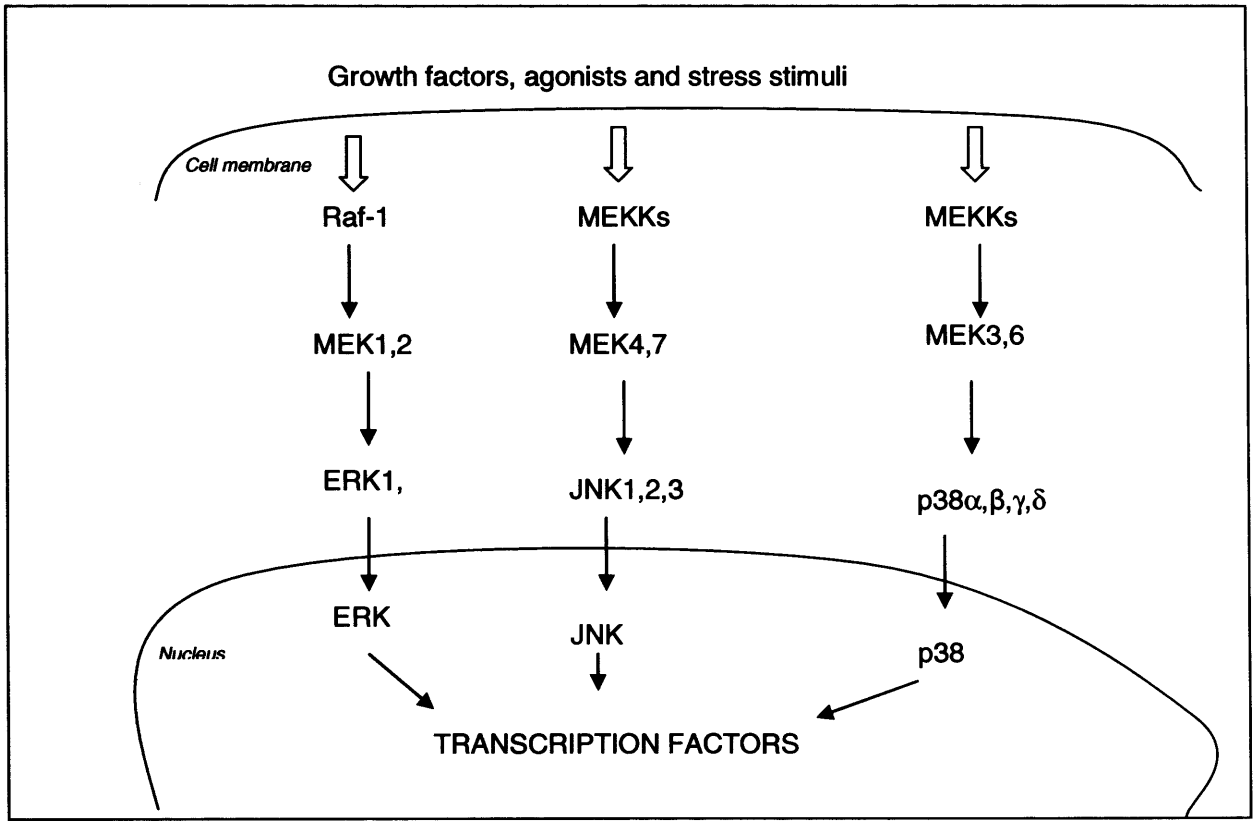


Figure 1.3 Schematic representation of the extracellular signal regulated kinases. Various stimuli induce activation of membrane bound receptors that activate MEKKs that in turn activate MEKs resulting in phosphorylation of ERK, JNK or p38. Activated ERK, JNK and p38 translocate to the nucleus and activate a number of transcription factors.

All 4 types of MAPKs have been implicated in modulation of apoptosis in cardiomyocytes after ischaemia reperfusion injury (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001) and will be discussed in more detail in turn.

1.3.1.1 Extracellular Signalling Regulated protein Kinases (ERKs) and Big Mitogen Activated protein kinases (BMK-1/ERK-5)

ERKs have been shown to be involved in cardioprotection in the heart (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001). Inhibition of ERKs by PD98059 increased the number of apoptotic rat neonatal cardiomyocytes after anthracycline exposure (Zhu et al., 1999). More importantly inhibition of ERKs augmented the injury caused by ischaemia/reperfusion in rat neonatal cardiomyocytes and rat perfused hearts (Yue et al., 2000). Studies on transgenic mice that were heterozygous for ERK 2 have shown enhanced infarction areas after ischaemia/reperfusion whereas transgenic mice with activated MEK1-ERK1/2 in the heart were largely resistant to ischaemia/reperfusion injury. In addition to infarct areas the amount of DNA laddering and TUNEL positivity was also reduced in the MEK1 transgenics compared to littermate controls after ischaemia reperfusion injury (Lips et al., 2004).

Although, not much is known about the role of BMK1 in cardiomyocytes, it has been shown to be important in cardiac development, as $ERK5^{-/-}$ mice have

malformed hearts and die on embryonic day 10.5 (Regan et al., 2002). Work on cell lines has revealed that ERK5 is also antiapoptotic. PC-12 cells exposed to oxidative stress demonstrated increased amount of apoptosis after inhibition of BMK-1 (Suzaki et al., 2002). In similar work, overexpression of dominant negative BMK-1 in MEF-7 cells resulted to dose dependant cell death and cell death augmentation after oxidative stress (Weldon et al., 2002).

1.3.1.2 Jun N-terminal Kinases

Oxidative stress in adult rat hearts has been linked with activation of JNK; JNK activation leads to cell death which can be diminished by overexpression of dominant negative SEK-1, an upstream activator of JNK (Aoki et al., 2002). Overexpression of constitutively active SEK-1 led to induction of apoptosis (Aoki et al., 2002).

Administration of antisense constructs of JNK-1 and -2 to perfused hearts has shown that JNK-1 and not -2 is responsible for induction of apoptosis in the heart after ischaemia reperfusion injury (Hreniuk et al., 2001).

1.3.1.3 P38 Kinases

Similar to JNK, p38 inhibition resulted in decrease in apoptosis and improved postischaemic cardiac function in perfused rat hearts and in rat neonatal cardiomyocytes (Ma et al., 1999; Mackay and Mochly-Rosen, 1999; Yue et al., 2000). Furthermore, administration of vanadate, an inhibitor of p38 MAPK phosphatase rendered rat neonatal cardiomyocytes more susceptible to ischaemia reperfusion injury in a dose dependant manner over time (Cong et al., 1997).

1.3.2 Protein Kinase B

Another Ser/Thr kinase that has an important function in regulating apoptosis is protein kinase B (PKB)/Akt. Akt is activated by insulin (Kulik et al., 1997), insulin-like growth factor-1 (Oh et al., 1998) and gp130 signalling (Mackay and Mochly-Rosen, 2000). Akt's function in attenuating apoptosis has been linked with its ability to phosphorylate and inactivate the proapoptotic gene BAD (Fig. 1.1) (Yaoita et al., 2000).

Insulin Growth factor treatment of cardiomyocytes resulted in activation of PI-3K and Akt and protected cells from apoptotic cell death after ischaemia reperfusion injury. Overexpression of dominant negative PI-3K and Akt attenuated apoptosis in this model of ischaemia reperfusion injury demonstrating the protective effects of the PI-3K/Akt pathway (Mackay and Mochly-Rosen, 2000). Hearts from transgenic mice overexpressing IGF-1 were more resistant to apoptotic cell death after ischaemia reperfusion. This was attributed to activated Akt, as wortmanin, an inhibitor of Akt, attenuated the resistance of the cardiomyocytes to ischaemia reperfusion injury (Yamashita et al., 2001). In a rat model of cardiac ischemia reperfusion injury *in vivo,* gene transfer by adenoviruses of constitutively active mutant Akt reduced infarct size, the number of apoptotic cells and prevented hypoxia-induced abnormalities in cardiomyocytes, calcium transients and shortening (Matsui at al., 2001).

1.3.3 Protein Kinase C

Protein kinase C is a family of Ser/Thr kinases. There are more than 16 isoforms of protein kinase C isolated from mammalian cells (Dempsey et al., 2000). Protein kinase C family members are involved in almost all aspects of cellular physiology, such as proliferation, mitogenesis, apoptosis, platelet activation, remodelling of actin cytoskeleton, modulation of ion channels, secretion, membrane permeability, cell migration, contraction and hypertrophy (Toker, 1998; Dempsey et al., 2000). PKC was initially discovered by Nishizuka and coworkers and soon after it was shown to be the main target of diacyl glycerol (DAG) and phorbol esters. PKC is activated by Ca^{+2}, phosphatidyl serine (PS) and DAG (Mackay & Mochly-Rosen, 2001).

The isoforms of PKC are divided into three subfamilies according to sequence similarities and activation profiles (Table 1.2):

1. Conventional PKCs (cPKCs) that include the isoforms α, β_I, β_{II} and γ.
2. Novel PKCs (nPKCs) including δ, ε, η and θ.
3. Atypical PKCs (aPKC) that have as members the ζ and λ (also known as ι) isoforms.
4. The recently described PKCs that include the isoforms μ (also known as protein kinase D) and ν.
5. Lastly in the PKC family there is a category of kinases included that are called Protein kinase C related kinases (PRK). The PRK1-3 are also known as PKN.

Table 1.2. Protein Kinase C subfamilies and activation profiles.

PKC Subfamilies	**Isoforms**	**Co-Factor**	**Activator**	**Lipid activators**
Conventional (cPKC)	α, β_I, β_{II}	Phosphatidyl Serine and Ca^{+2} dependancy	Diacyl gycerol	Phospho inositodes, PMA, Arachidonic acid, LPA, Lyso PC
Novel (nPKC)	δ, ε, η, θ	Phosphatidyl Serine, Ca^{+2} independent	Diacyl glycerol	Phospho and phosphatidyl inositodes, PMA, arachidonic acid, LPA, Lyso PC
Atypical (aPKC)	ζ, λ/ι	Phosphatidyl Serine	DAG independent	Phosphatidyl inositodes, Ceramide
Recently described	μ/PKD, ν	Phosphatidyl Serine	DAG independent	
PKC related kinases (PRK/PKN)	PRK1, PRK2, PRK3			

The PKC isoenzyme specificity is determined by their subcellular localisation. After activation each isoenzyme is translocated to a unique subcellular site, where it is anchored by specific proteins the RACKS.

The cPKCs are activated by PS in a Ca^{+2} dependent manner. They bind to and activated by DAG, which increases their specificity for PS that in turn increases their affinity for Ca^{+2} (Table 1.2).

nPKCs are activated by DAG and require PS as a cofactor, like cPKCs, but they have lost dependency to Ca^{+2} (Table 1.2).

aPKCs and the recently discovered PKCs do not respod to DAG or Ca^{+2}, but require PS as a cofactor (Table 1.2).

Several lipid second messengers have been shown to activate PKC family members. Activation of PLC, leads to production of 1, 2, sn diacyl glycerol (DAG) and phosphoinositodes InsP, or Ins-1, 4-P2, or Ins-1, 4, 4-P3. Phosphoinositodes lead to release of Ca^{+2} from intracellular stores that with DAG and PS induce a conformational change to PKC, which binds to the plasma membrane and becomes activated (Table 1.2). Other lipid second messengers have been shown to activate PKCs as well (Table 1.2). The PI-3K products Phosphatidyl Inositols PtdIns-3, 4-P2 and PtdIns-3, 4, 5-P3 have been shown to activate nPKCs and aPKCs. Arachidonic acid is able to contribute to PKC activation as well, but only

when DAG or phorbol 12-myristate-13-acetate (PMA) is present (Table 1.2). LPA and Lyso PC also activate PKCs in the presence of DAG (Table 1.2). Ceramide, which is produced by TNFa cell stimulation, is able to activate PKCζ and Raf-1 (Table 1.2).

PKC has been shown to be phosphorylated by the PI-3K dependent kinase PDK-1. PDK-1 phosphorylation releases PKC from the membrane and allows it to remain in the cytosol in an inactive form.

1.3.3.1 Protein Kinase C and apoptosis

Activation of PKC by PMA can be either pro- or anti- apoptotic depending on cell type (Lucas and Sanchez-Margalet, 1995).

Overexpression of a PKCα dominant negative construct was shown to induce apoptosis in COS-1 cells (Whelan and Parker, 1998) and in salivary gland cells (Dempsey et al., 2000). $PKC\beta_{II}$ was protective against c-myc induced apoptosis in small lung carcinoma cells (Barr et al., 1997). PKCλ/ι and PKCζ protect K562 leukemia cells (Murray and Fields, 1997) and UV irradiation decreased the activity of PKCζ and PKCλ/ι (Berra et al., 1997).

Inhibition of PKCδ in salivary gland epithelial cells suppresses caspase activation and DNA fragmentation (Reyland et al., 1999). Dominant negative PKCδ overexpression inhibited phorbol ester induced apoptosis in prostate cancer cells

(Fujii et al., 2000). Also, proteolytic activation of PKCδ by caspases produces a catallyticaly active fragment in cells induced to undergo apoptosis by DNA damaging agents (Emoto et al., 1996).

1.3.3.2 Protein Kinase C in ischaemia reperfusion injury

Cardiac myocytes express at least six different PKC isoenzymes (Disatnik et al., 1994), but preconditioning results in activation of only PKCδ and PKCε (Gray et al., 1999). Preconditioning is a phenomenon observed in cardiac cells, where short periods of ischaemia are able to minimise the amount of cell death occurring after a major ischaemic incident that follows those short periods of ischaemia. So the cell seems to become "conditioned" to ischaemia through brief ischaemic episodes.

PKC α and ε were shown to be translocated after periods of hypoxia in cardiomyocytes, whereas the amount of membrane associated PKCδ was reduced (Goldberg et al., 1997). PKCα and ε were also shown to be membrane associated after brief periods of ischaemia and reperfusion in rat hearts (Albert and Ford, 1999).Introduction of PKCε selective inhibitory peptide eV1-2 into neonatal cardiomyocytes prevented protection from preconditioning, whereas inhibitors of other PKC isoenzymes had no effects (Gray et al., 1999). A PKCε selective translocation agonist caused significant reduction in ischaemia induced cell death of neonatal and adult cardiomyocytes (Dorn et al., 1999). When PKCε was introduced as a transgene in mouse hearts there was a faster recovery and a significant reduction in cardiac damage after prolonged no flow ischaemia

(Dorn et al., 1999). Also in isolated cardiomyocytes, perfused hearts and transgenic mice the PKCδ and ε were shown to have opposing effects on protection with the latter to have cardioprotective effects (Chen et al., 2001). Experiments with constitutive active constructs of PKCδ and ε have shown that their opposing effects on apoptosis are due to a differential regulation of extracellular regulated kinases. PKCε was shown to be an activator of p42/44 and having no effects on p38 and JNK, whereas PKCδ had exactly the opposite effects (Heidkamp et al., 2001).

1.3.3.3 Protein Kinase C in hypertrophy

PKC δ and ε have both been shown to be involved in induction of hypertrophy (Chen et al., 2001). Endothelin-1, an inducer of hypertrophy, was shown to result in translocation of PKC δ and ε, whereas phenylephrine treatment only induced PKCε translocation. In both cases, the translocation of the PKC isoforms resulted in MAPK p42/44 activation (Clerk et al., 1994). Another study has shown that PKC α, but not β_I, δ, ε or ζ induces hypertrophy as shown by increase in cell size, ANF expression and [H^+] Leucine incorporation. Overexpression of dominant negative PKCα blocked hypertrophy. Furthermore, PKCα increased ERK1/2 activation, whereas dominant negative PKCα reduced activation of ERK1/2 by PMA and dominant negative MEK1 inhibited PKCα induced hypertrophy (Braz et al., 2002).

1.3.4 Phospholipases

The phospholipases are a large and diverse group of enzymes with a wide variety of biological functions, from membrane synthesis to generation of signalling molecules. Phospholipases have been shown to be involved in hypoxia reoxygenation in the heart and as such they are good candidates for further research in their role in hypoxic injury in the heart.

Phospholipases are categorised into four classes according to the position of the hydrolytic cleavage of their substrate. The four classes and the position of the hydrolysis are schematically represented on Fig. 1.3.1.

1.3.4.1 Phospholipase A_2

Phospholipases of the A class are involved in membrane integrity and the production of biological active molecules. They are further divided into two categories: PLA_1 and PLA_2. PLA_2 are able to hydrolyse the sn-2 bond pf phospholipids and produce Lysophospholipids and fatty acids. PLA_2s are classified into three groups:

1. cytosolic PLA_2 ($cPLA_2$)
2. Ca^{+2}-dependent secretory PLA_2 ($sPLA_2$). They are of low molecular size with a dependency on milimollar quantities of Ca^{+2} and
3. Ca^{+2}-independent intracellular PLA_2 ($iPLA_2$).

They are of high molecular weight (85kD), are able to translocate to membranes in response to increments of intracellular Ca^{+2} and are specific for phospholipids that contain arachidonic acid on sn-2

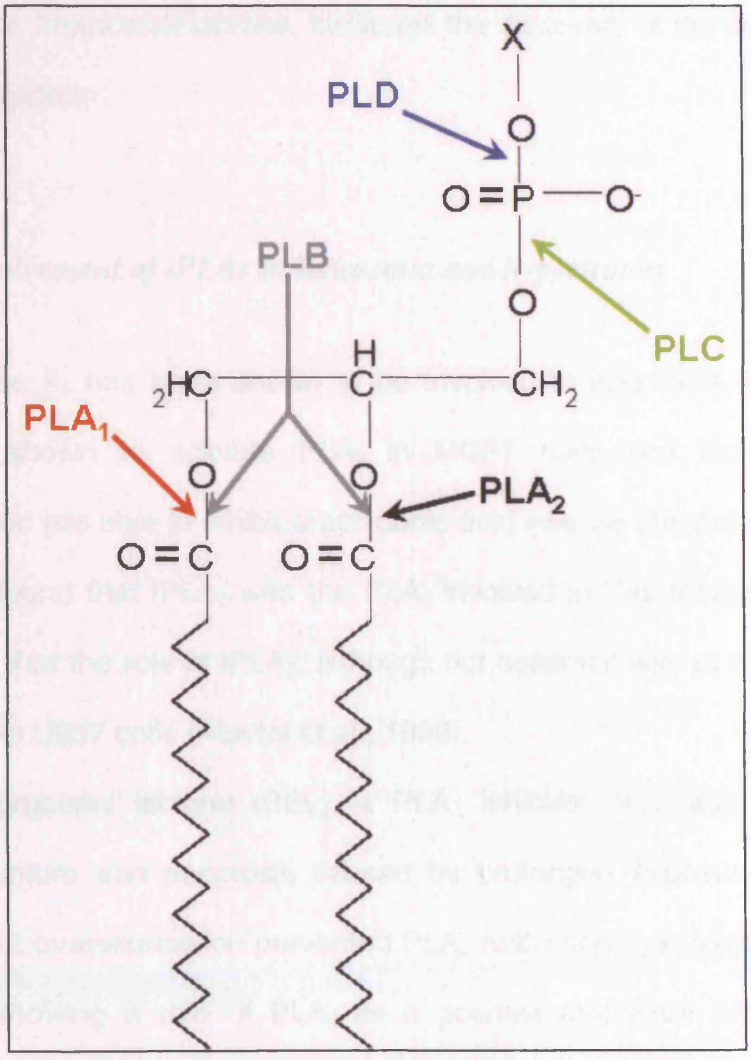


Figure 1.3.1. Hydrolytic sites of the different phospholipases on a glycerol phospholipid. PLA_1 and PLA_2 cleave at the sn-1 and sn-2 positions, respectively, to generate a lysophospholipid and a free fatty acid. PLB enzymes cleave at both the sn-1 and sn-2 positions. PLC hydrolyses the bond between the glycerol and phosphate, often generating important signalling molecules, such as diacylglycerol and phosphoinositodes. PLD removes the polar head group (x) to generate phosphatidic acid. (Adapted from Brown et al., 2003).

Cyclic AMP (cAMP), PKC and MAPK have been shown to decrease the activity of PLA_2, either by direct phosphorylation or by regulation of PLA_2 inhibitory proteins, such as lipocortins and annexins.

We will study the effects of $iPLA_2$ in more detail, as the existence of a well used $iPLA_2$ inhibitor, bromoenol lactone, facilitates the discovery of the possible role of $iPLA_2$ in our system.

1.3.4.1.1 Involvement of iPLAs in ischaemia and hypertrophy

Phospholipase A_2 has been shown to be involved in apoptosis. Fas and TNF have been shown to activate PLA_2 in MCF7 cells and Bcl-2 and Bcl-x overexpression are able to inhibit arachidonic acid release (Jaattela et al., 1995). It was later found that $iPLA_2$ was the PLA_2 involved in Fas mediated fatty acid release, and that the role of $iPLA_2$, although not essential was at least modifying in apoptosis in U937 cells (Atsumi et al., 1998).

Similarly, bromoenol lactone (BEL), a PLA_2 inhibitor, was able to attenuate lysosomal rupture and apoptosis caused by prolonged exposure to H_2O_2. In addition, Bcl-2 overexpression prevented PLA_2 activation, lysosomal rupture and cell death, showing a role of PLA_2 as a positive modulator of cell death in oxidative stress (Zhao et al., 2001a; Zhao et al., 2001b).

Likewise, in RAW264.7 cells and in mouse peritoneal macrophage cultures, $iPLA_2$ was shown to be the form of phospholipase involved in oxdative stress (Martinez and Moreno, 2001). Only $iPLA_2$ was shown to be active after H_2O_2

sress. Furthermore, the arachidonic acid release, due to increased activity of $iPLA_2$, was dose dependent to H_2O_2. Arachidonic acid release by H_2O_2 was blocked by BEL. In addition, antisense $cPLA_2$ had no effect on the arachidonic acid release showing that $iPLA_2$ was the isoform activated by H_2O_2 (Martinez and Moreno, 2001).

Recently, it was shown that $iPLA_2$ is crucial for caspase-independent cell death. Purified PLA_2 induced nuclear shrinkage in permeabilised cells and PLA_2 inhibitors prevented hypoxic nuclear shrinkage and cell death. Hypoxia caused elevation of PLA_2 activity and translocation to the nucleus. Knockdown of $iPLA_2$ delayed nuclear shrinkage and cell death (Shinzawa and Tsujimoto, 2003).

A number of experiments have shown the involvement of PLA_2 in ischaemia reperfusion injury in hearts (Prasad et al., 1991; Schwertz and Halverson, 1992; Sargent et al., 1996; Vesterqvist et al., 1996). Interestingly, although BEL was able to increase coronary flow and decrease lactose dehydrogenase release during reperfusion, the presence of 5 hydroxy decanoate (a K_{ATP} inhibitor) in the perfusate during reperfusion abolished the cardioprotective effects of BEL, linking K_{ATP} channel cardioprotection with PLA_2 (Sargent et al., 1996).

Vassopressin induced arachidonic acid release in H9c2 cells (of cardiac origin), caused an influx of Ca^{+2} and activation of PKC $\alpha,\beta I,\varepsilon$ and δ. PKC was shown in turn to be able to modulate arachidonic acid release through a MAPK pathway, creating a positive feedback loop (Chen and Chen, 1999). Also, $iPLA_2$ was shown to be involved in lethal malignant tachyarrhythmias during acute cardiac ischaemia (Mancuso et al., 2003).

There is not much evidence for involvement of PLA_2 in hypertrophy, however Haq et al. (2003) shown that cytosolic PLA_2 is a negative regulator of growth, specifically of striated muscle. Normal growth of skeletal muscle and normal and pathologic stress-induced hypertrophic growth of the heart are exaggerated in Pla2g4a -/- mice, which lack the gene encoding cytosolic PLA_2. They were able to show that absence of PLA_2 leads to sustained activation of IGF-1, which results from the failure of PDK-1 to recruit and phosphorylate PKCζ, a negative regulator of IGF-1. Arachidonic acid addition was able to restore activation of PKCζ, correcting IGF-1 signalling.

In conclusion, $iPLA_2$ is an important modulator of cell death following a hypoxic insult and it has a considerable role in cardiac hypertrophy.

1.4 Ubiquitination

Ubiquitination or ubiquitylation is a type of protein modification, where a small protein, ubiquitin, is attached covalently to other proteins. We are interested in the process because ubiquitination of a protein may lead to its destruction and it is part of the apoptotic process. As we will show later on, number of enzymes involved in the ubiquitination process, like the inhibitors of apoptosis (IAPs) are also involved in the apoptotic pathway.

Ubiquitin is a 76 amino acid globular protein that is highly conserved from yeast to human. The process of ubiquitination is essential for the degradation of proteins whose levels are regulated either constitutively, or in response to the cellular environment. As such, ubiquitination, is involved in most if not all of cellular processes such as cell cycle progression, organelle biogenesis, apoptosis, cellular differentiation, protein transport, inflammation, antigen processing, DNA repair and stress responses (Weissman, 2001).

Ubiquitination is a multistep process (Fig. 1.4.1), involving at least three types of enzymes. First, an ubiquitin activating enzyme (E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP dependant process. Then a ubiquitin-conjugating enzyme, or ubiquitin-carrying enzyme (UBC or E2) accepts ubiquitin from the E1 enzyme by a trans-thiolation reaction, again involving the carboxy terminus of ubiquitin. Finally, an ubiquitin protein ligase (E3) catalyses the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the substrate (Hochstrasser, 1996; Pickart, 2000; Weissman, 2001; Di Fiore et al., 2003).

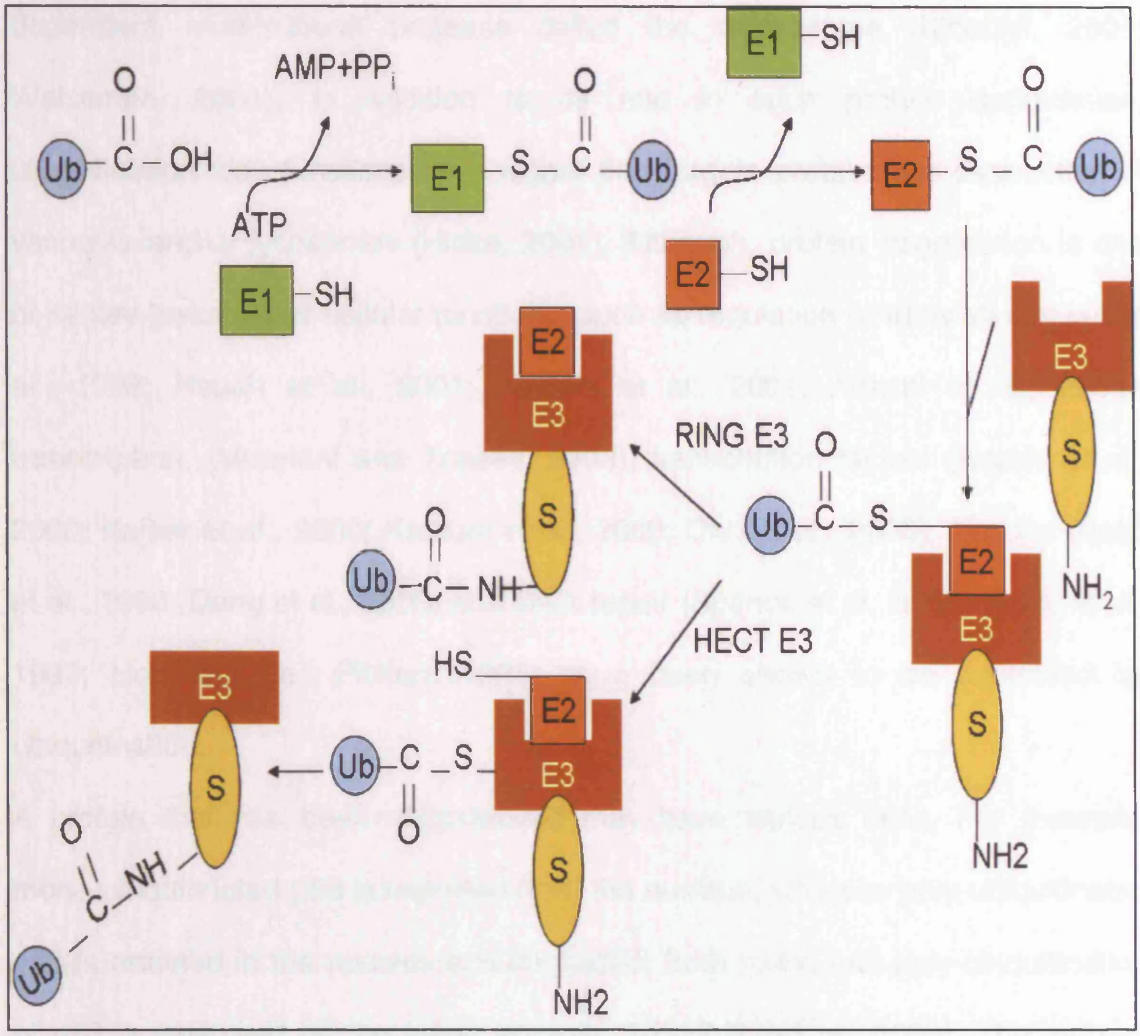


Figure 1.4.1. The ubiquitination pathway. Free ubiquitin (Ub) is activated by an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s which might or might not have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the active site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate bound multi-ubiquitin chain. For RING E3s, current evidence suggests that ubiquitin might be transferred directly from the E2 to the substrate. Adapted from Weissman, 2001.

Ubiquitination is traditionally linked with protein degradation by an ATP-dependent multi-subunit protease called the proteasome (Kloetzel, 2001; Weissman, 2001). In addition to its role in such protein degradation, ubiquitination also functions as a signal that targets proteins for destruction in vacuoles and/or lysosomes (Hicke, 2001). Although, protein degradation is one of its key tasks, other cellular functions, such as regulation of translation (Liao et al., 1998; Hsueh et al., 2001; Kentsis et al., 2001; Adachi et al., 2004), transcription, (Muratani and Tansey, 2003), transcription factors (Hoppe et al., 2000; Kaiser et al., 2000; Kamura et al., 2000; Ohh et al., 2000), kinases (Baldi et al., 1996; Deng et al., 2000) and DNA repair (Spence et al., 1995; Bailly et al., 1997; Hofmann and Pickart, 1999) have been shown to be controlled by ubiquitination.

A protein that has been ubiquitinated may have various fates. For example, mono-ubiquitinated p53 is exported from the nucleus, whereas poly-ubiquitinated p53 is retained in the nucleus and degraded. Both mono and poly-ubiquitination of p53 is catalysed by the same enzyme, Mdm2 (Li et al., 2003). The authors suggest that the level of ubiquitination is determined by the levels of Mdm2 in a dose dependant manner. This example shows that ubiquitination is a versatile signal.

There are many levels of regulation that influence the specificity of the process. Specificity is largely generated by the proteins that recognise the substrates and mediate ubiquitination. But, as in the example above, the fate of the ubiquitinated protein is influenced by the type of the conjugate formed. Thrower et al. have

shown that mono-ubiquitinated substrates are not destroyed by the proteasome, and that the shortest signal for recognition is a tetra-ubiquitin chain (Thrower et al., 2000). In addition, there are different ways of building a poly-ubiquitin chain, by using different lysine residues of ubiquitin. Also, intracellular location helps to determine the fate of the ubiquitinated protein.

Proteins can also be modified by the addition of small proteins other than ubiquitin. There is a growing list of ubiquitin like proteins (UBLs) that have been identified and characterised. As with ubiquitin, UBLs include a glycine at the carboxy terminus that forms an isopeptide bond with the ε-amino group of lysines on the target protein. Of the UBLs that are homologous to ubiquitin the very first to be characterised was a protein that resembles a ubiquitin dimer, the ubiquitin cross-reactive protein (UCPR or ISG15) (Loeb and Haas, 1994). RUB1 from yeast, which stands for related to ubiquitin, also belongs in this group, and it is known as Nedd8 in metazoans. Other UBLs include SUMO-1 (small ubiquitin-related modifier, also known as UBL1), Sentrin or PIC-1 and Apg12 (Jentsch and Pyrowolakis, 2000; Yeh et al., 2000). Apg12 is a UBL that lacks amino acid homology with ubiquitin. Apg12 is involved in a multienzyme process that resembles ubiquitination and mediates autophagy (Ohsumi, 2001).

In addition to UBLs, an increasing number of structurally unrelated proteins are found that contain domains homologous to ubiquitin. This ubiquitin domain proteins (UDPs) have a number of functions and are not known to be involved in protein modification. In the UDP group there are proteins that are ubiquitination substrates and proteins involved in the addition and/or the removal of ubiquitin

from proteins. Some UDPs interact with the proteasome as well as with enzymes that are involved in mediating ubiquitination (Yeh et al., 2000).

Further specificity is introduced by the presence of deubiquitinating enzymes (DUBs) that cleave ubiquitin from residual proteasome associated peptides and from multiubiquitin chains (Weissman, 2001). DUBs are also important for processing immature ubiquitin, which is encoded on multiple genes and translated as fusion proteins either with other ubiquitin molecules or as the amino terminal component of two small ribosomal subunits (Finley et al., 1989). These are processed by a subfamily of DUBs called the ubiquitin carboxy-terminal hydrolases.

1.4.1 E1 Ubiquitin activating Enzymes

E1 is encoded by a single gene, which produces two isoforms, through alternative translation start sites (Handley-Gearhart et al., 1994). The localisation of the longer isoform is cell cycle regulated and its levels in the nucleus reach a maximum in G2 phase (Grenfell et al., 1994).

The carboxy terminal glycine of ubiquitin, SUMO-1 and UBLs is essential for activation by E1 (Yeh et al., 2000). However, SUMO-1 and UBLs require their own E1-like enzymes (Yeh et al., 2000). E1s and E1-like enzymes share homology at their amino- and carboxy- terminals.

1.4.2 E2 Ubiquitin Conjugating Enzymes

E2s are extremely diverse. There are at least 13 E2s (Ubc1-13) in yeast and at least 25 mammalian counterparts (Weissman, 2001). Of these Ubc 9 is dedicated to sumoylation and Ubc 12 to rubylation. Sumoylation, rubylation and nedylation are protein modifications similar to ubiquitination with the difference that SUMO-1, RUB-1, or Nedd-8 is covalently attached to the modified protein instead of Ubiquitin. E2s share an approximately 35% homologous core of 14-16 kD (Weissman, 2001). Their role is to receive Ubiquitin from E1 enzymes and transfer them to E3s.

1.4.3 Types of E3 ubiquitin Ligases

There are two main types of E3 ubiquitin ligases. The HECT E3s and the RING finger E3s.

1.4.3.1 HECT E3s

The oncogenic strains of the Human Papillomaviruses (HPV) encode a protein called E6 that interacts with and inactivates the tumour suppressor protein p53. It was found that E6 acts as an adaptor for a cellular protein, called E6 associated protein (E6-AP), that catalyses the ubiquitination of p53 (Scheffner et al., 1993). The characterisation of E6-AP led to the discovery of a protein family that contains a highly conserved 350 amino acid domain at their carboxy termini called the HECT domain (for Homologous to the E6-AP carboxy terminus). This

includes a cysteine that forms a covalent thiol-ester intermediate with ubiquitin (Huibregtse et al., 1995).

Another feature that is shared among many HECT E3s is the WW domain, which is involved in protein-protein interaction. It is characterised by a pair of tryptophans 20-22 amino acids apart, and an invariant proline within a region of 40 amino acids. WW domains interact with proline rich regions (Kay et al., 2000). Most WW HECT E3s contain another domain at the carboxy terminus, the C2 domain that mediates translocation to the plasma membrane in response to increases in intracellular calcium (Plant et al., 2000).

1.4.3.2 RING finger E3s

The RING finger motif was first identified in the RING1 gene and was categorised as a type of Zn finger motif with DNA binding abilities (Freemont et al., 1991, Lovering et al., 1993). However, it was first linked with ubiquitination when it was observed that PRT1 (Proteolysis 1) protein, an N-end rule E3[1] from *Arabidopsis thaliana,* shared the RING finger motif with other proteins implicated in ubiquitination (Potuschak et al., 1998). Since then, most of the RING finger proteins that have been identified are implicated in ubiquitination.

[1] The half life of a protein is governed by a rule that states that the metabolic stability of a protein is determined by the identity of its N-terminal residue. The N-terminal rule exists in prokaryotic and eukaryotic cells alike. Those signals that exist in the N-terminal of proteins targeted for destruction are called N-degrons and the machinery that recognises the N-degrons and orchestrates the proteolytic destruction of the degron-containing proteins, forms the N-end rule pathway. In eukaryotes the N-end rule pathway is part of the ubiquitin system (Varshavsky, 1996).

1.4.3.2.1 Structure of RING Finger

There are two consensus sequences for the RING finger motif: $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}CX_2CX_{(4-48)}CX_2C$ and $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}HX_2CX_{(4-48)}CX_2C$ (Weissman, 2001).

The Cys and His residues have Zn binding ability and the difference between the two types of motifs is on the amino acid of the fifth coordination site. When this is occupied by a Cys the RING finger is of the RING HC type and in the case of a His occupying the site it is of the RING H2 type. A schematic representation of the RING finger structure is given in figure 1.4.2.

The RING finger E3s can be further divided into two categories:

1. The Single subunit E3s and
2. The multisubunit E3s.

The single subunit E3s contain the substrate recognition element and the RING finger on the same polypeptide. Multisubunit E3s contain a small RING finger protein and a member of the cullin family of proteins, as well as other subunits (Weissman, 2001).

1.4.3.2.1.1 Single subunit E3s.

Single subunit E3s include the Mdm2 protein, which ubiquitinates p53, the proto-oncogene c-Cbl, which ubiquitinates growth factor receptors and the inhibitors of apoptosis (IAPs) (Weissman, 2001). Parkin is also a RING finger E3 that

contains two RING motifs at its carboxy terminus separated by an IBR (in-between RING), a region common to proteins that contain 2 RING motifs (Mizuno et al., 2001; Tanaka et al., 2004). Parkin also has an amino-terminal ubiquitin domain, making it a member of both the RING and UDP families. Mutations in Parkin's RING are associated with juvenile Parkinson's disease, and a synaptic vesicle associated protein (CDCrel-1) has been identified as a substrate for this E3 (Mizuno et al., 2001; Tanaka et al., 2004).

The RING finger is found in a number of diverse and unrelated proteins. So the sites of substrate recognition for RING proteins must be highly varied. The interactions of c-Cbl depend on its atypical SH2 domain and for the IAPs probably on the BIR domain. For Mdm2, interactions with p53 occur through its amino-terminal domain, whereas the RING finger lies on the carboxy terminus (Weissman, 2001).

1.4.3.2.1.2 Multisubunit E3s.

There are three main types of multisubunit E3s:

1. The Skp1-Cullin-F-box complex (SCF complex),
2. the von Hippel-Lindau-Cul2/ElonginB/Elongin C (VCB-CUL2 complex), and
3. the Anaphase Promoting Complex (APC) (Fig. 1.4.3).

All three complexes contain a small RING finger protein and a member of the cullin family (proteins with homology to Cul1, which was first shown to be involved in the cell-cycle exit in *C. elegans).* It appears that cullins act as a

backbone that interacts with linker proteins that recruit the substrate recognition components (Fig. 1.4.3).

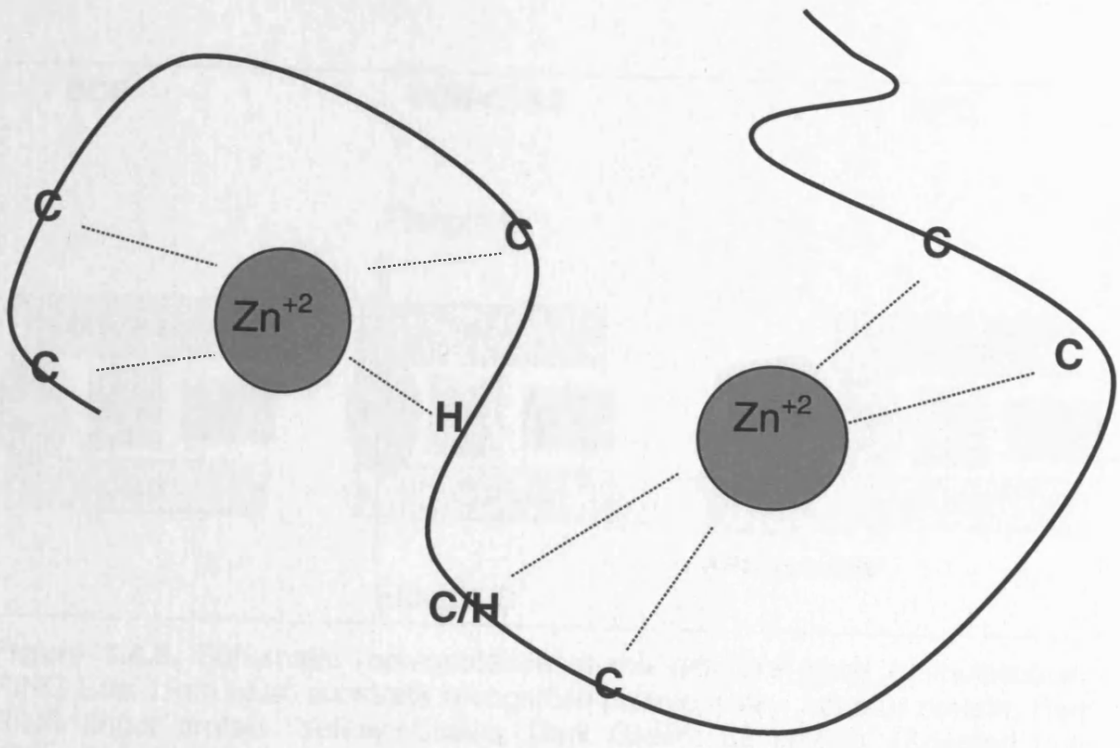


Figure 1.4.2. Schematic representation of the structure of the RING finger motif. C and H denote the Zinc binding amino acids.

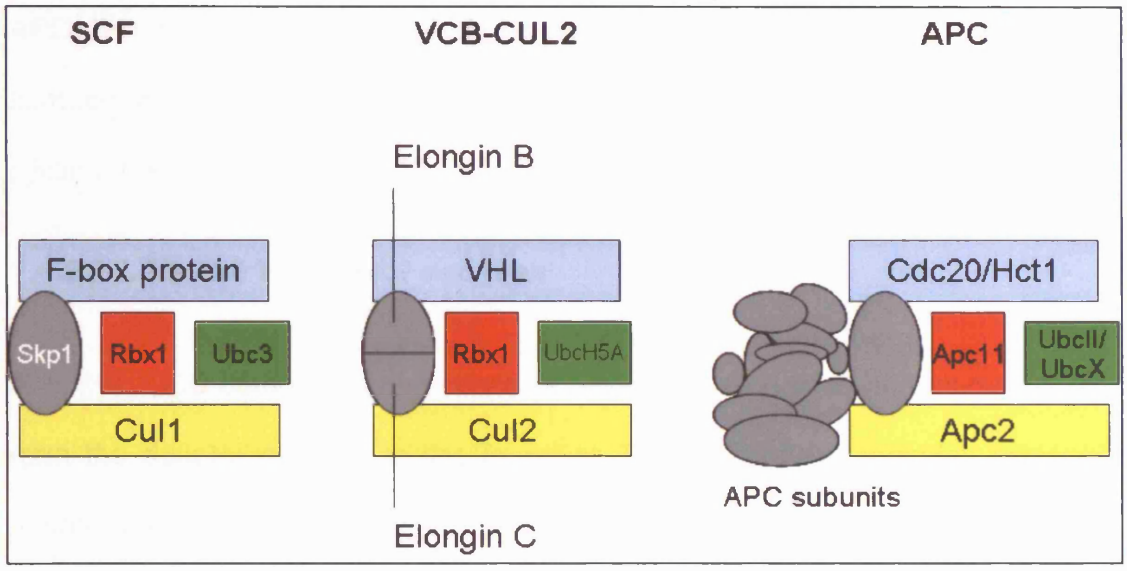


Figure 1.4.3. Schematic representation of the different types of multisubunit RING E3s. Light blue: substrate recognition protein, Grey: adaptor protein, Red: RING finger protein, Yellow: Cullins, Dark Green: E2 protein. (Adapted from Weissman, 2001).

1.4.3.2.1.2.1 The Anaphase Promoting Complex

APC is the most intricate of the three, composed of at least 12 different components in the yeast. The first identified substrates for APC were mitotic cyclins, but other substrates have now been identified (Weissman, 2001).

1.4.3.2.1.2.2 The VCB-CUL2 complex

The VHL-CBC complex is composed of a dimer of elongin B and elongin C that form the adaptor proteins in this complex. The substrate recognition protein is mainly VHL, and mutants of VHL that fail to bind to the CBC core have been associated with malignacies in the von Hippel-Lindau disease (Iwai et al., 1999; Lisztwan et al., 1999). Hypoxia inducible factor 1a (HIF1a), a regulator of vascular endothelial growth factor (VEGF), has been identified as a substrate for this E3 (Kamura et al., 2000). The VHL protein contains a suppressor of cytokine signalling box (SOCS-box) that interacts with the core of this E3 (Kamura et al., 1998). SOCS-1 has been shown to act as a substrate recognition protein on a multisubunit E3 that regulates the half life of Vav and JAKs (Kile et al., 2002).

1.4.3.2.1.2.3 The SCF complex

SCF E3s recognise and ubiquitinate a large amount of phosphoproteins. The substrate recognition is made through proteins that contain a F-box motif and Skp1 is the main adaptor protein of the complex. One F-box protein is able to recognise more than one substrate, as is the case with the beta-Transducin-repeat-containing protein (βTRCP) (Fuchs et al., 2004). $SCF^{\beta TRCP}$ is able to

recognise both IκBα and β-catenin (Fuchs et al., 2004). In addition, nascent forms of HIV bound CD4 are indirectly targeted for degradation in the endoplasmic reticulum membrane by $SCF^{\beta TRCP}$ as the HIV encoded Vpu protein has phosphorylation sites similar to those of β-catenin and IκBα (Besnard-Guerin et al., 2004; Fuchs et al., 2004). Some F-box proteins are themselves ubiquitinated and targeted for degradation. This might be a way of autoregulating their levels. In addition, another SCF protein, Met30, has been shown to regulate the activity of Met4, a transcription factor. Multiubiquitination of Met4, that occurs in minimal media in yeast leads to protein degradation, whereas oligo-ubiquitination of Met4 in rich medium, leads to recruitment of Met4 on SAM gene promoters, rather than MET gene promoters and has no effect on the stability of the transcription factor (Kuras et al., 2002).

1.4.4 The Sensitive to Apoptosis Gene

One RING finger gene that was recently identified was named Sensitive to Apoptosis Gene (SAG) and was cloned as a factor that is induced after oxidative stress (Duan et al., 1999). SAG is a 13 kD protein, containing a $C_2H_2C_4$ Zn-RING finger motif (Fig. 1.4.4). It was recently shown to be expressed in hypoxic conditions in the brain and to co-localise with newly produced radical oxygen species (ROS), indicating a possible function for SAG as an antioxidant. The RING finger motif of SAG has been shown to be important for its antiapoptotic function, as mutants of the SAG at the RING motif are unable to protect from apoptosis when overexpressed (Ohta et al., 1999a).

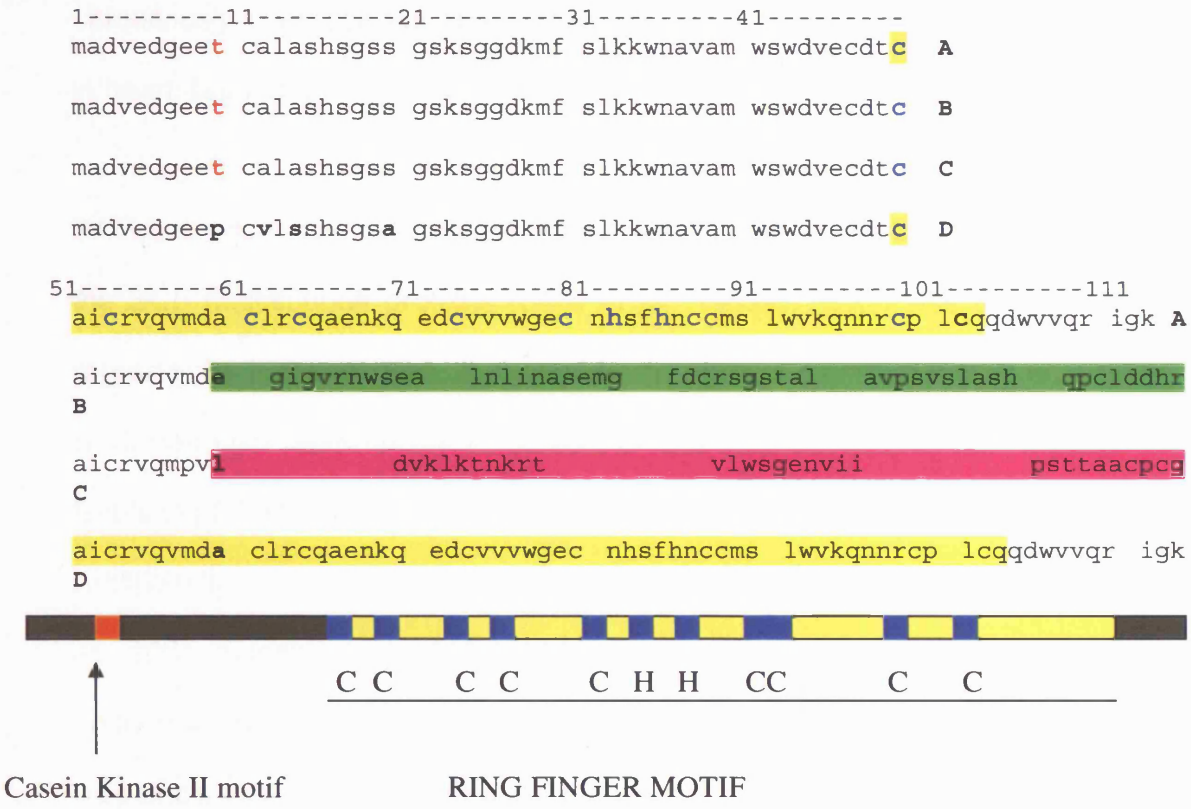


Figure 1.4.4. Sequence alignment and schematic representation of the Sensitive to apoptosis gene proteins. A Sequence of Human SAG, B and C possible SAG isoforms resulting from alternative splicing in humans and D murine sequence of SAG protein. Amino acids in bold are non homologous aminoacids. Amino acids highlighted with red are site of protein phosphorylation by CKII, with yellow comprising the RING finger motif, with blue important for Zn^{+2} binding, with green and pink parts of the protein resulting from alternative splicing.

SAG was shown to be localised in the cytoplasm and nucleus of the cell and is ubiquitously expressed in many different organs and tissues, but predominantly in heart, brain and the gonads (Duan et al., 1999).

SAG belongs to the regulator of Cullins (ROC) family, that bind through cullin to the SCF multisubunit proteins (Ohta et al., 1999a). ROC1 has been shown to regulate the levels of Sic1, the yeast homologue of $p27^{Kip1}$ (Ohta et al., 1999b). SAG has also been shown to be part of SCF box complexes that regulate the levels of p27 (Duan et al., 2001).

Additionally, SAG is overexpressed in several carcinomas (Sun, 1999; Huang et al., 2001; Sasaki et al., 2001), although over-expression of SAG alone is not enough to induce neoplastic transformation of cells (Huang et al., 2001). SAG was shown to promote S-phase and serum free growth of cells (Duan et al., 2001) and down regulation of SAG, by transient transfection of an antisense SAG construct in cancer cells resulted in reduction of the proliferation rate of these cells (Huang et al., 2001) demonstrating the importance of SAG in control of cell proliferation. Furthermore, the involvement of SAG in cell proliferation was demonstrated in yeast, where it was shown by gene chip profiling to regulate expression of components of the cell cycle (Sun, 1999; Swaroop et al., 2000).

As SAG is shown to be an antiapoptotic factor that is expressed in the heart and that is upregulated by oxidative stress, there is a good possibility to have a role in hypoxic stress in cardiac cells.

1.5 Ion Channels in the cardiomyocytes

The mechanical activity of the heart is controlled by electrical impulses. The electric impulses in the heart are a result of series of ion currents through the cardiac cell membrane that are regulated by specific ion channels. Any effect on an ion channel is bound to be met with serious effects on the cardiac cell physiology. For this reason we need to examine the ion channels of the heart in more detail.

The electric impulses are intrinsic to the heart, but are also modulated by neuronal activity through the autonomic nervous system. Specialized cells in the right atrium, known as the sino-atrial node, act as a pacemaker and the impulses are conveyed to all the atrial myocytes, leading to a coordinated depolarisation and contraction of the atria. On a surface electrocardiogram (ECG) atrial depolarisation can be visualised as a P wave (Fig. 1.5.1). The impulse from the sino-atrial node is also conveyed to specialised cells that connect the atria to the ventricles, known as the atrio-ventricular node. Then the electrical activity is conveyed to specialised fibres known as bundle branches, leading to rapid coordinated contraction of ventricular myocytes and coordinated contraction of the ventricles (Keating and Sanguinetti, 2001). Depolarisation of the ventricles can be visualised on the ECG as the QRS complex (Fig. 1.5.1). Ventricular myocytes slowly repolarise, which is denoted on the ECG as the T wave (Fig. 1.5.1), leading to cardiac relaxation and completion of one cardiac cycle (Keating and Sanguinetti, 2001).

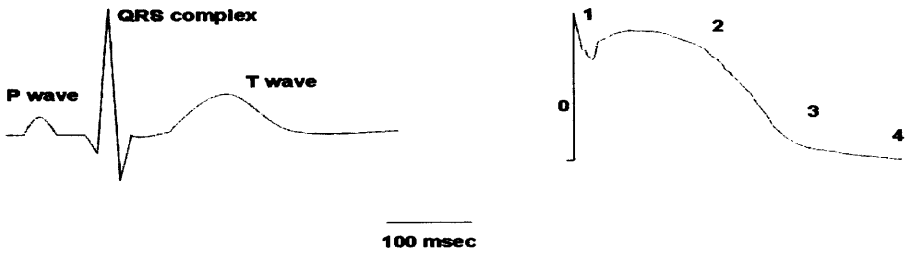


Figure 1.5.1 Schematic representation of a typical electrocardiogram (left) and the action potential in the ventricle (right).

In a prototypical fast response cell, the membrane is highly permeable to K^+. This permeability reflects the fact that inward rectifier K^+ channels in the membrane are open at rest (Keating and Sanguinetti, 2001). By contrast, the resting membrane is Na^+ impermeant. The cardiac sodium channels in resting cells are closed. A change in the membrane potential across the cell is sensed by the Na^+ channels which alter their conformation and open allowing a large rapid Na^+ influx, producing the typical rapid phase 0 depolarisation (Keating and Sanguinetti, 2001) (Fig. 1.5.1) .

In some cells a rapid phase 1 repolarisation then occurs, because of outward K^+ movement via transient outward channels. During phase 0 and 1, Ca^{+2} channels open. Phase 2 is the characteristically long plateau phase of the cardiac action potential and reflects a balance between inward current through mainly L-type Ca^{+2} channels and outward current, largely through delayed rectifier K^+ channels. The net outward current during phase 3 repolarisation is produced by delayed rectifier channels along with inactivation of Ca^{+2} channels (Keating and Sanguinetti, 2001). Final repolarisation during phase 4 is accomplished by outward movement of K^+ through inward rectifier channels (Keating and Sanguinetti, 2001).

1.5.1 Potassium channels

Potassium channels are a diverse group of membrane proteins that are found on excitable and non-excitable cells and are involved in a variety of processes, such as neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction and cell volume regulation (Shieh et al., 2000). The role of these membrane proteins is to conduct K^+ across the membrane. To accomplish this all potassium channels are characterised by the following (Shieh et al., 2000):

1. The pore: a water filled permeation pathway that allows K^+ to flow across the membrane.
2. Selectivity filter: specifies K^+ as permeant ion species and
3. Gating mechanism: allows the channel to switch between open and close conformations.

Potassium channels can be categorised into three groups according to the number of putative transmembrane segments (Shieh et al., 2000).

A. The six-transmembrane-one pore channel,

B. the two-transmembrane-one pore channel and

C. the four-transmembrane-two pore channel

1.5.1.2 Inward Rectifying Potassium channels (Kir)

The ATP-sensitive potassium (K_{ATP}) channels belong to the ATP-binding cassette transporter superfamily and are comprised of two subunits:

1. a pore-forming, inward-rectifying potassium subunit (Kir) that belongs to the two-transmembrane-one pore potassium channel group, and
2. a regulatory sulfonylurea receptor (SUR).

The Kir and SUR subunits coassemble with a 4:4 stoichiometry to form a hetero-octameric K_{ATP} channel with the Kir subunits forming the K^+ permeation pathway (Shieh, et al., 2000) (Fig. 1.5.2). Both Kir and SUR subunits are required to form functional channels with the SUR subunit cooperating with the Kir subunit to act as an ATP-dependent potassium channel complex. The SUR subunit contains two transmembrane segments and two nucleotide binding folds (NBD-1 and NBD-2) (Fig. 1.5.2.). The NBDs are located near the carboxy terminus of the SUR subunit and contain the Walker A and B motifs, essential in providing membrane sensitivity to ATP and Mg^{+2}-ADP. The Walker A motif contains the characteristic glycine-rich motif $GlyX_6GlyXXGlyXGlyLys(Ser/Thr)$ allowing binding of ATP or GTP by means of the terminal phosphate group (Shieh, et al., 2000). The Walker B motif contains the characteristic amino acid sequence $ArgX_{11}AspX_6Asp$ and is involved in ATP hydrolysis and binds the Mg^{+2}-ATP (Shieh, et al., 2000). The nucleotide binding fold NBD-1 has limited catalytic activity, whereas NBD-2 hydrolyses ATP and has been shown to play an essential role in K_{ATP} channel gating. Both NBD-1 and -2 are required for K_{ATP} channel function (Shieh, et al., 2000).

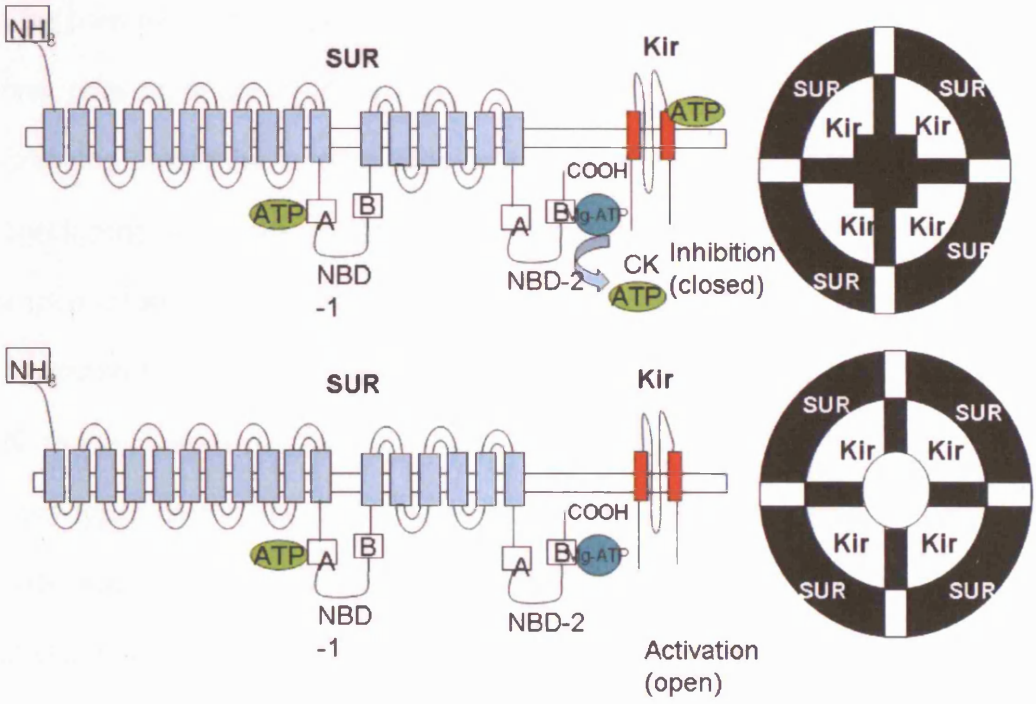


Figure 1.5.2 Schematic representation of K_{ATP} channel. Two dimensional representations of pore forming inward rectifying potassium channel subunit (Kir) and a regulatory sulfonylurea receptor (SUR) during closed and open states. Nucleotide binding folds, containing Walker A and Walker B motifs are shown (NBD-1 and 2). Under homeostatic conditions K_{ATP} channels are closed. High ATP concentrations allow binding of ATP to Kir, closing the pore. Binding of ATP to NDB-1 and hydrolysis of Mg^{+2}-ATP at NBD-2 allows limited resident binding of Mg^{+2}-ADP to NBD-2 due to catalytic actions of Creatine Kinase (CK), thus preventing channel opening. Under conditions of ischaemia ATP concentrations are decreased and ADP concentrations are increased and the catalytic actions of CK are reduced or inhibited and resident binding of Mg^{+2}-ADP to NBD-2 is increased, allowing conformational stabilisation and K_{ATP} channel opening and K^+ flux across the inner membrane.

The pore forming inward rectifying K^+ channel belong to the two transmembrane one pore category of K^+ channels. They are comprised of two transmembrane domains linked by a pore loop in between (Fig. 1.5.2). These channels are conducting K^+ more in an inward rather than an outward fashion and are important in setting the resting membrane potential. This inward rectification is attributed to gating mechanisms by Mg^{+2} and polyamines that occlude access of K^+ to the internal vestibule of a conducting pore (Shieh, et al., 2000).

Two types of Kir and three types of SUR have been identified as part of K_{ATP} channels, Kir 6.1 and Kir6.2 and SUR1, SUR2A and SUR2B. It has been shown that the two Kir can combine to form functional channels in HEK293 cells (Cui et al., 2000). In the myocardium, two sensitive ATP channels have been isolated, one subtype in the sarcolemma and the other in the inner membrane of the mitochondria. The cardiac sarcolemmal channels have been characterised as SUR2A/Kir6.2. The Kir6.2 subunit has a molecular mass of 51kD, whereas the SUR2A subunit has a molecular mass of 140kD (Shieh, et al., 2000). The mitochondrial channel has yet to be molecularly characterised.

The K_{ATP} channels can be pharmacologically modulated by a family of structurally diverse agents collectively known as potassium channel openers and blockers. Most channel openers are non-selective and act through binding to the SUR subunits. The potassium channel openers are thought to act by interaction or by active competition at regulatory ATP binding sites in the K_{ATP} channel, allowing conformational stabilization through interaction with a conserved amino acid motif. In contrast potassium channel blockers act to inhibit conformational

stabilisation (Shieh, et al., 2000). The best known potassium channel blocker is glibenclamide which acts on both sarcolemmal and mitochondrial K_{ATP} channels. It binds to a high affinity site localised on any SUR subunit and with a low affinity on the Kir subunit (Cui et al., 2000; O'Rourke, 2000; Shieh, et al., 2000).

The selectivity of sarcolemmal and mitochondrial K_{ATP} channel openers and blockers is primarily dependent upon SUR phenotype and SUR subunit sensitivity. Pinacidil, cromakalim, nicorandil and related analogues are potassium openers that have been shown to act preferentially on SUR2A/B subunits and have relatively little effect on SUR1 containing K_{ATP} channels. Potassium channel blockers, act in a very similar manner. Recombinant and in vitro studies, however, have shown that the mitochondrial K_{ATP} channels can be selectively blocked with 5-hydroxy-decanoate (5-HD) (Cui et al., 2000; O'Rourke, 2000; Shieh, et al., 2000).

1.5.2.1.2 K_{ATP} channel and cardioprotection

Several studies have shown that the K_{ATP} channel is implicated in cardioprotection after iscaemia/reperfusion injury, because blockers like glibenclamide attenuate protection and the use of openers confer protection (Garlid et al., 1997, Liu et al., 1998, Kevelaitis et al., 1999, Baines et al., 1999, O'Rourke, 2000; Pain et al., 2000; Sato et al., 2000; Xu et al., 2001; McCully and Levitsky, 2003; Oldenburg et al., 2003; Baczko et al., 2004). In addition, there is no conclusive evidence for the role of sarcolemmal K_{ATP} channels in cardioprotection (O'Rourke, 2000; Pain et al., 2000; Sato et al., 2000; Xu et al., 2001; McCully and Levitsky, 2003; Oldenburg et al., 2003; Baczko et al., 2004).

There are several possible mechanisms suggested as to how K_{ATP} channels can protect against ischaemic injury. Mitochondrial swelling induced by mitochondrial K_{ATP} opening might improve mitochondrial energy production. Pharmacological opening of mitochondrial K_{ATP} improves oxidative phosphorylation during ischaemia reperfusion and this effect is inhibited by 5-hydroxy-decanoate or glibenclamide (Tanonaka et al., 1999; Iwai et al., 2000; Miura et al., 2000).

Another mechanism might be through an effect on Ca^{+2} handling. K_{ATP} channel openers decreased the rate of Ca^{+2} uptake by isolated mitochondria and a similar effect was observed in intact neonatal myocytes. 5-hydroxy-decanoate inhibited that effect (Holmuhamedov et al., 1999; Baczko et al., 2004). Depolarisation of

the mitochondrial membrane potential ($\Delta\Psi_m$) was thought to be responsible for the decrease in the rate of Ca^{+2} entry. However, another study showed that depolarisation of $\Delta\Psi_m$ by activation of K_{ATP} was not accompanied by changes in Ca^{+2}, and they rather attributed the protective effects of K_{ATP} opening to prevention of cytochrome c release from the mitochondrion (Xu et al., 2001).

A third possible mechanism suggests that the protection is conveyed through alteration of the rate of reactive oxygen species (ROS) release from the mitochondrion. ROS have long been implicated in the cellular damage associated with ischaemia and reperfusion (von Harsdorf et al., 1999a). However, ROS generation has been considered as a trigger in ischaemic preconditioning, a phenomenon where brief series of ischaemia, confer protection against infarction, prior to a prolonged period of ischaemia (Murry et al., 1986). In embryonic myocytes both protection and ROS production were inhibited by 5-hydroxy-decanoate, suggesting that the opening of K_{ATP} channels could stimulate ROS production (Yao et al., 1999; Oldenburg et al. 2003). ROS production can in turn activate protein kinase C, which can influence mitochondrial K_{ATP} activity (Sato et al., 1998; Sasaki et al., 2000; Huh et al., 2001; Liu et al., 2002).

The involvement of K_{ATP} channels in hypertrophy is as yet unclear. However, evidence suggests that K_{ATP} channels might have some kind of involvement in hypertrophy. K_{ATP} has been shown to be a potent regulator of ANF secretion (Xu

et al., 1996) and K_{ATP} channel opening inhibited stretch induced ANF release in atrial cardiomyocytes (Jiao et al., 2000). However more work is needed to further elucidate the possible role of K_{ATP} in cardiac hypertrophy.

1.6 The Corticotropin Releasing Factor family of peptides

In this chapter I am providing background information for the peptides whose effects in the cardiac cell we are studying.

The Corticotropin-Releasing Factor family consists of a number of structurally related peptides that include CRF, Urocortin (UCN), urotensin and sauvagine in vertebrates and the diuretic peptides present in insects (Lovejoy and Balment, 1999). Recently, two new homologues have been cloned from mice that show high similarity to UCN; they are UCNII and UCN III (Lewis et al., 2001; Reyes et al., 2001). At the same time a different group cloned the human homologues of UCN II and III, which they named Stress Related Peptide (SRP) and Stresscopin (SCP), respectively (Hsu and Hsueh, 2001). Henceforth, the murine homologues will be refered to as UCNII and III and the human homologues as SRP and SCP respectively.

1.6.1 Synthesis, structure and tissue distribution of CRF and UCN

The CRF gene is located on chromosome 8 and the UCN gene on chromosome 2, in humans. Both genes consist of two exons and the entire coding region for both peptides is located in the second exon (Zhao et al., 1998). Both proteins are produced by cleavage of the C-terminus of a pre-pro-peptide (Vale et al., 1997). The mature peptides are of similar size consisting of 41 aminoacids for CRF and 40 for UCN.

The human and rat CRF are identical, but they differ to the ovine CRF by seven amino acids (Lovejoy and Balment, 1999) (Fig. 1.6.1). The amphibian peptide sauvagine (SVG) and the fish peptide urotensin (URO), share approximately 50% homology to the human and rat CRF (Lovejoy and Balment, 1999) (Fig. 1.6.1).

UCN is more highly conserved among species than CRF and is closely related to fish URO (53-63% amino acid homology) and diverges from CRF (43-45% homology) and SVG (35% homology).

CRF mRNA is widely expressed in the central nervous system, with major sites of expression in the paraventricular nucleus of the hypothalamus, the cerebral cortex, the cerebellum and the amygdala-hippocampal complex (Bittencourt and Sawchenko, 2000). In the periphery, CRF is expressed in the adrenal gland, the testis, placenta, gut, spleen, thymus and skin (Vale et al., 1997).

UCN was originally identified in the rat midbrain, and has been shown to be expressed in the central nervous system, mainly in the Edinger-Westphal nucleus, hypothalamus, lateral superior olive cerebellum, substantia nigra pars compacta and ventral tegmental area (Skelton et al., 2000). In the periphery, UCN is expressed in the vasculature, the digestive system, the adrenal gland, placenta, lung, thymus, spleen, in leucocytes and the heart. In the heart, UCN is expressed in both myocytes and non-myocytes, as well as in vascular smooth muscle cells of the cardiac blood vessels, with highest concentration detected in

the left ventricle (Leitch et al., 1998 and Kimura et al., 2002). CRF has not been detected in the heart.

Although, CRH and UCN activate both type 1 and 2 receptors[2], the lack of a pervasive UCN projection to CRH receptor 2 expressing cells (Bittencourt et al., 1999) and the absence of CRH or UCN projections to brain anxiety centers (Weninger et al., 1999) pointed to the existence of additional CRH related peptides.

Following this hypothesis SRP/UCN II and SCP/UCNIII were cloned in humans and mice. Human SCP encodes a prepro-protein of 161 aminoacids and a putative mature protein of 40 amino acids (Hsu and Hsueh, 2001). Human SRP on the other hand encodes a prepro-protein of 112 amino acids that gives rise to a predicted 43 amino acid mature peptide (Hsu and Hsueh, 2001).

The open reading frames of hSCP and hSRP contain a signal peptide for secretion and the predicted mature regions are flanked by potential proteolytic cleavage sites and an α-amidation donor residue. However, the human SRP precursor lacks an obvious C-terminal proteolytic cleavage site and has two uncommon cysteine residues flanking the predicted mature peptide, suggesting that new proteolytic enzymes, yet undiscovered, might be responsible for SRP maturation (Hsu and Hsueh, 2001).

---

[2] For more information on CRH receptors please refer to chapter 1.6..4

Although, the CRH family shares similar secondary structures, the predicted structures of mature SCP and SRP are distinct from those of other family peptides at the N-terminus. The mature CRH, UCN, URO and SVG all share a similar structure with an N-terminal random coil, followed by an extended α-helical structure. In contrast, the N-terminal sequence of SCP, SRP, their two pufferfish orthologs and murine UCNII adopted an extended strand, followed by a short random coil (Hsu and Hsueh, 2001).

The SCP transcript has been shown to be abundantely expressed in the brain, colon, small intestine, muscle, stomach, thyroid, adrenal gland, pancreas, heart, kidney and spleen (Hsu and Hsueh, 2001; Takahashi et al., 2004). SRP has been shown to be expressed mainly in the brain, adrenal gland, heart and peripheral blood cells (Hsu and Hsueh, 2001).

Sequence homology analysis shows that there is only a 37% homology between SCP and SRP, 42% between SCP and murine UCNII, 26% with human UCN and SCP and 26% between human CRH and SCP (Hsu and Hsueh, 2001). Additionally, phylogenetic analysis of 10 CRH family proteins from fish and mammals indicated an ancient evolution of three subgroups of CRH family proteins with the human and pufferfish SCPs clustered in a separate branch from human SRP and murine UCNII (Hsu and Hsueh, 2001).

1.6.2 CRH Peptide family evolution

It is becoming well clear now that the vertebrate genomes contain four distinct genes of the CRH family:

1. CRH
2. UCN/URO
3. SCP/UCNIII and
4. SRP/UCNII.

Each of these genes is highly conserved during evolution (Chang and Hsu, 2004).

Phylogenetic analysis shows that the origin of each of these peptides predates the evolution of tetrapods and teleosts, spanning an evolutionary history of 550 milion years (Fig. 1.6.2) (Chang and Hsu, 2004). It appears that this family of peptides evolved from an ancestral gene that developed the CRF/UCN and SCP/SRP branches through an early gene duplication (Fig. 1.6.2) (Chang and Hsu, 2004).

Peptide	Sequence	Length	Identity (%)
hCRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII	41	100
oCRF	SQEPPISLDLTFHLLREVLEMTKADQLABQAHSNRKLLDIA	41	83
URO	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV	41	54
hUCN	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	40	43
SVG	ZGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI	40	48
hSRP	IVLSLDVPIGLLQILLEQARARAAREQATTNARILARV	38	34
mUCNII	VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV	38	34
hSCP	FTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI	38	32
mUCNIII	FTLSLDVPTNIMNILFNIDKAKNLRAKAAANAQLMAQI	38	26

Fig. 1.6.1. Alignment of members of the Corticotropin releasing factor family. The amino acids that are homologous between the CRF family are boxed. Human stresscopin related peptide (SRP) and human urocortin II (UCN II) are identical. Human stresscopin and human urocortin III are also identical. Abbreviations: h: human, o: ovine, m: murine, SVG: sauvagine, URO: urotensin I. Adapted from Dautzenberg and Hauger, 2002.

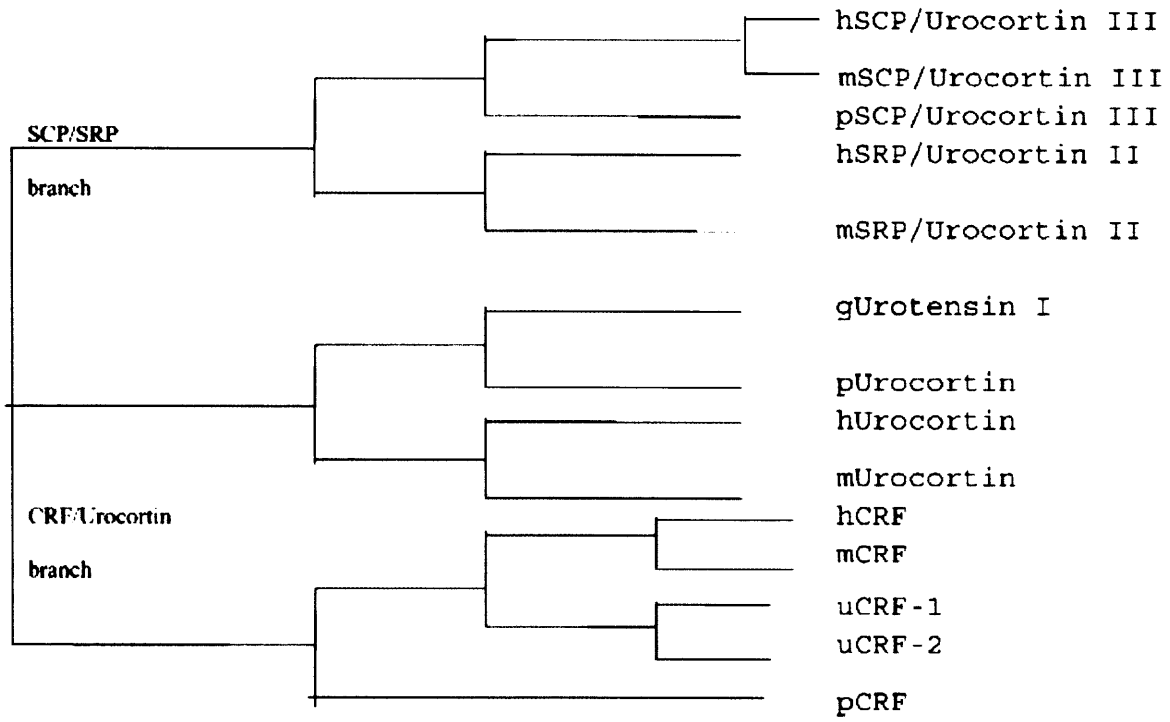


Fig. 1.6.2. Evolution of the CRH family of peptides. The four vertebrate family peptides probably derived from an ancestral gene through gene duplication. During the first gene duplication, two ancestral genes, a SCP/SRP like gene and a CRH/UCN like gene, were created. A second round of gene duplication generated the four core ligand peptides in vertebrates. H: human, m: mouse, p: puffer fish (*Fugu rubripes*), u: white sucker (*Catostomus commersioni*), g: goldfish (*Carassius auratus*). Adapted from: Chang and Hsu, 2004.

1.6.3 Actions of CRF peptides

CRF and related peptides have been implicated in the regulation of the stress responses of organisms, by mediating the release of adrenocorticotropin (ACTH) from the anterior pituitary (Lovejoy and Balment, 1999; Skelton et al., 2000; Reyes et al., 2001). In addition, the CRF family of peptides has been implicated in the regulation of the cardiovascular system (Nishikimi et al., 2000; Parkes and May, 2000; Parkes et al., 2001; Terui et al., 2001), the gastrointestinal system (Kihara et al., 2001; Okamoto et al., 2001; Shade et al., 2002) and inflammatory processes (Kohno et al., 2001; Radulovic and Spiess, 2001).

CRF administration increases blood pressure, cardiac output and heart rate and CRF perfused through isolated rat hearts can increase coronary flow and maximal aortic pressure (Parkes and May, 2000).

Urocortin administration in fasted rats stopped gastroduodenal motility and provoked fed-like patterns (Kihara et al., 2001). Leptin a protein that decreases food intake was shown to be mediated by CRF as injection of anti-CRH attenuated the effects of leptin aministration in rats (Okamoto et al., 2001). Intracerebroventricular injections of CRH in baboons significantly reduced the daily food intake in the subject animals (Shade et al., 2001).

UCN increased IL-1 beta and IL-6 secretion by human peripheral blood mononuclear cells and CRH receptor immunoreactivity was strong in

mononuclear inflammatory cells of rheumatoid arthritis synovia, suggesting that acts as an immune-inflammatory mediator (Kohno et al., 2001).

1.6.4 CRF receptors

The functions of the CRF peptides are elicited by binding to G protein-coupled receptors (Grammatopoulos et al., 2000). Two families of receptors are known thus far: CRF receptor type 1 (CRFR1) and CRF receptor type 2 (CRFR2). There is further redundancy in the CRFR2 family, as there are so far three splice variants or subtypes cloned named CRFR2α, CRFR2β and CRFR2γ (Kostich et al., 1998). The two receptors have different anatomical distribution, different pharmacology and different affinities for the various CRF family peptides. In rodents both receptors are expressed in the central nervous system (CNS), but their peripheral distribution differs. CRFR1 is expressed in the gonads and the skin, whereas CRHR2β (a splice isoform of CRFR2) is mainly expressed in the epididymis, the gastrointestinal tract and in cardiac and skeletal muscle. CRFR2α, the other splice variant is expressed mainly in the brain (Perrin and Vale, 1999).

Comparisons of the binding affinities and potencies of the known UCN peptides to CRFR1, CRFR2α and CRFR2β stably expressed on CHO cells showed that UCN II and III exclusively bind to type 2 receptors whereas UCN I seems to be able to bind to both types albeit with a higher affinity to type 2a (Lewis et al., 2001). In contrast, CRF has a 10 fold higher affinity for the type 1 receptor (Vaughan et al., 1995).

This difference of ligand affinity of the receptors and their different anatomical distribution strengthens the possibility that CRF and UCN may have different functions. Indeed, the two types of receptor have been associated with the different functional aspects of the CRF peptide family. Thus, the type 1 receptors have been implicated with anxiogenic-type behaviours as shown by experiments on CRHR1 knock-out mice (Tazi et al., 1987; Smith et al., 1998). As far as the CRHR2 type is concerned, until recently, due to the lack of knock-outs and antagonists that specifically block this type of receptor, it has been difficult to attribute specific functions to it. However, following behavioural studies on CRHR2 knock-out mice, it is suggested that the type 2 receptor mediates a general anxiolytic response, as the knock-out mice were more susceptible to stressful behaviour than the wild type ones (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000). In addition, knock-out mice exhibited larger edema responses in response to thermal injury than their wild type littermates (Kishimoto et al., 2000), validating the main role of CRHR2 in the peripheral responses of the CRH family of peptides.

1.6.5 UCN cardioprotective and hypertrophic effects.

UCN has been shown to protect cardiac myocytes from cell death after exposure to hypoxia/reoxygenation injury *in vitro* (Okosi et al., 1998; Brar et al., 1999; Brar et al., 2000). In addition, UCN has similar effects in the isolated perfused rat heart (Brar et al., 2000). As CRFR2β is the only subtype expressed in the rat heart (Nishikimi et al., 2000), the cardioprotective effects of UCN against hypoxia/reoxygenation (Okosi et al., 1998; Brar et al., 1999; Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al., 2002b; Latchman, 2002) are attributed to the binding to the CRRFR2. In more detail, UCN has been shown to decrease mean arterial blood pressure and increase heart rate (Terui et al., 2001; Parkes et al., 1997; Abdelrahman and Pang, 2003) and in addition increase aortic flow and cardiac contractility (Parkes et al., 1997). Similarly, UCNII has been shown to also induce a dose dependent reduction in mean arterial blood pressure and increase in heart rate when given intravenously in rats (Mackay et al., 2003). Moreover, it has been shown that UCN is able to protect neonatal cardiac myocytes (Okosi et al., 1998; Brar et al., 1999; Brar et al., 2000; Brar et al., 2002b; Railson et al., 2002), adult cardiomyocytes (Brar et al., 2002b; Railson et al., 2002) and whole hearts either *in vitro* (Brar et al., 2000; Lawrence et al., 2002; Scarabelli et al., 2002b; Schulman et al., 2002) or *in vivo* (Schulman et al., 2002) from hypoxia-reoxygenation injury.

However, UCN has also been shown to be involved in cardiac hypertrophy. UCN is able to increase distinct markers of hypertrophy, such as protein synthesis, cell size, atrial (ANP) and brain (BNP) natriuretic peptide secretion and collagen in cardiomyocytes (Ikeda et al., 1998; Nishikimi et al., 2000; Railson et al., 2002). In addition, expression of UCN mRNA was higher in human hearts with left ventricular hypertrophy (Nishikimi et al., 2000) and UCN immunoreactivity was more intense in cardiomyocytes of human failing hearts, than in normal human hearts (Nishikimi et al., 2000). However, in sheep with experimental heart failure, ovine UCN administration, increased cardiac output, while arterial peripheral resistance and mean arterial pressure fell. Furthermore, it induced dose dependent decreases in plasma vasopressin, natriuretic peptides and endothelin-1 (ET-1) (Rademaker et al., 2002), showing that, although UCN clearly induced hypertrophic markers, the overall systemic effect of the peptide administration improved the viability of the sheep with heart failure, indicating that more work is needed to elucidate the balance of its cardioprotective effect and its effect on hypertrophy.

We have previously shown that cardioprotection from hypoxia/reoxygenation injury induced by UCN and its homologues in rat neonatal cardiomyocytes is dependent on activation both of Akt and MAPK p42/44 as inhibiton of Akt and p42/44 pathways by chemical inhibitors, or with overexpression of dominant negative constructs abolished cardioprotection by the peptides (Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al., 2002b; Railson et al., 2002; Schulman et al., 2002; Brar et al., 2004a; Brar et al., 2004b).

In addition, it was shown that the MAPK p42/44 pathway is not involved in the manifestation of the hypertrophic effects of UCN in rat neonatal cardiomyocytes, as transfection of cardiomyocytes with a dominant negative MEK1 construct, prior to UCN administration, failed to attenuate the increase in mean cell area by UCN (Railson et al., 2002). It was previously postulated that the increase in cAMP by UCN administration is mediating its hypertrophic effects (Nishikimi et al., 2000; Rademaker et al., 2002). However, Akt activation has also been implicated in cardiac hypertrophy (Frey and Olson, 2003), but its role in the hypertrophic effects of UCN has not been studied.

1.7 AIMS

In our work we have tried to identify and compare the effects of stresscopin and stresscopin related peptide to urocortin on cardioprotection and hypertrophy.

We wanted to identify whether the newly identified UCN homologues are expressed in the rat cardiomyocytes and if ischaemia had any effects on its expression; also we studied and compared the effects of the peptides on cell death and apoptosis after hypoxia reoxygenation, in order to identify any differences on the potency of the three peptides (Chapter III).

We also wanted to examine the effects of the three peptides on primary neonatal cardiomyocyte cell size, protein synthesis and expression of the natriuretic peptides, in order to determine whether the three peptides have any hypertrophic effects (Chapter IV).

The role of MAPK and Akt in cardioprotection and hypertrophy by the UCN homologues was examined (Chapter V).

The role of three signalling molecules PLA_2, PKC_ε and Kir 6.1 in cardioprotection by the three homologues, recently indentified to be involved in the UCN signalling pathway, was examined (Chapter VI).

Lastly, I examine whether the three homologues affect the expression of SAG. In addition we investigated whether SAG is able to protect cardiomyocytes from hypoxia/reoxygenation injury and look further into a possible role for SAG in the cardiomyocytes (Chapter VII).

CHAPTER II

MATERIALS AND METHODS

2.1 Preperation of rat neonatal cardiomyocytes

2.1.1 Materials

Digestion buffer

- 100mls ADS

60mg ~250u/mg collagenase type II CLS2 (Worthington)

25mg pancreatin from porcine pancreas (Sigma #P-3292)

- **500mls ADS Buffer**

500mls MilliQ H_2O

3.4g NaCl

2.38g HEPES

0.06g NaH_2PO_4

0.5g Glucose

0.2g KCl

0.05g MgSO4

Adjust to pH 7.35 and filter sterilize.

- Foetal Calf Serum (FCS)
- **Plating Media**

DMEM (Dulbeco's Modified Eagle's Medium)

15% FCS

1X Penicylin/Streptomycin

- **Maintenance media**

DMEM

1% FCS

1X Pen/Strep

- **1% Gelatin in PBS**

PBS

1% Gelatin

Boil gelatin and filter sterilize

2.1.1.2 Method

1. Weigh out 60 mg collagenase and add to 100 ml ADS. Also, weigh out 25 mg pancreatin.
2. Oxygenate buffer using cylinder in primary TC during heart isolation.

Heart Isolation

1. For isolation take ADS buffer in petri dish and 1L of 70% EtOH.
2. Cull pups, rinse in 70% EtOH, isolate hearts into ADS buffer.
3. In primary TC dissect hearts with scissors.

Serial Digestion

1. Add 25 mg pancreatin to oxygenated ADS and filter sterilize.
2. Pipette hearts into 25 cm^2 TC flask and remove buffer using pipette.
3. Rinse hearts once with 10 ml digestion buffer, let hearts settle and discard buffer. Add 10 ml of fresh digestion buffer and incubate at 37°C for 15min.
4. Gently shake flask, let the hearts settle and pipette out buffer. Discard this first digestion.
5. Repeat digestion and following incubation add buffer to 2 ml of FCS in 15 ml falcon (as soon as this is done add another 10mls of digestion buffer and begin the next digestion) and centrifuge @1000rpm for 5min. Discard supernatant, re-suspend in 4 mls FCS and incubate in 37°C.
6. Repeat digestion for a total of 8 times. (There are 9 altogether including the first that is discarded).

Pre-plating

Calculate volume of plating media required. Cells are plated at $1x10^6$/ml and approximately 4.5 hearts yield enough for 1 plate (6 or 24 wells), which is 24mls.

1. To pre-plate use 175 cm^2 TC flasks, up to 200 ml per flask. Measure out plating media into flasks.

2. Pool the eight myocyte suspensions and centrifuge @1000rpm for 5min. Discard supernatant and re-suspend in 20 ml 15% plating media. Pipette equal volumes of cell suspension to pre-plating flasks and incubate at 37°C for 1h.

Plating

1. Coat TC plates with 1% gelatin (in PBS) at 37°C for 1h.
2. Remove gelatin.
3. Pool cells into one 175 cm^2 flask and plate cells. Gently shake flask after every few plates so all the cells haven't settled to the bottom.
4. Allow overnight for cells to attach and the following morning replace media with 1% maintenance media.

2.2 Simulated ischaemic insult

Prior to induction of simulated ischaemia cells were washed twice with phosphate buffered saline (1xPBS) and fresh Esumi modified Ischaemic Medium (Brar et al., 1999) was added to the cells which were transferred to a gas chamber for induction of hypoxia in an atmosphere of 95% Argon and 5% CO_2 for the desired amount of time. After completion of the hypoxic treatment, cells were washed twice with 1xPBS and Esumi Modified control medium (Brar et al., 1999) was added. Cells were then transferred to the incubator for up to 16 hours in an atmosphere of 21% O_2 and 5% CO_2. Control cells were maintained in these conditions in an incubator for the duration of the experiment in Esumi modified control medium.

2.3 Langendorff Perfused Rat Hearts

Hearts from anaesthetized adult male Sprague-Dawley rats were mounted in a Langendorff perfusion apparatus, and ischemia/reperfusion was performed as described previously (Scarabelli et al., 1999; Brar et al., 2000). Isolated hearts were divided into 3 groups. The first group contained control hearts that were continuously perfused. The second group was exposed to 35 min of ischaemia alone, which was achieved by occlusion of the left coronary artery. The hearts in the last group were reperfused for 60 min, after 35 min of ischaemia. Heart samples were frozen on liquid nitrogen immediately at the end of the experiment and protein extracted from the tissues as described previously (Brar et al., 1999).

2.4 RNA isolation

Total RNA was isolated from $5x10^6$ cardiomyocytes that were washed with 1xPBS prior to being lysed in 1 ml of TRIzol (GIBCO BRL, Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA, USA). 100µl of chloroform was added to the lysate and the mixture centrifuged at 13000 rpm for 15 min on a table top centrifuge. The aqueous phase was transferred to a fresh tube and an equal amount of isopropanol was added, mixed and incubated on ice for 30 min. After centrifuging as above the pellet was washed with 70% ethanol and left to dry prior to resuspending in DEPC treated H_2O. The amount of RNA was determined by spectrophotometry and the samples stored at -80°C.

2.5 cDNA preparation

First strand synthesis was done using 1 μg of total RNA, and 1 μl of oligo-dT primers (Promega UK Ltd, Southampton, UK). AMV reverse transcriptase (Promega UK Ltd, Southampton, UK) was used according to the manufacturer's instructions. Semi-quantitative PCR was performed as described before (Smith et al., 2001) using Imolase polymerase (Bioline Ltd, London, UK) and the following primers

SAG forward: 5'-CTCGAGATGGAGGACGGCGAGGAA-3'

SAG reverse: 5'-GGTACCCTCTCATTTGCCGATTCT-3'

Actin forward: 5'-TCATGAAGTGTGACGTTGACATCCGT-3'

Actin reverse: 5'-CCTAGAAGCATTTGCGGTGCACGACGATG-3'.

The PCR products were visualised on 2% polyacrylamide gel and analysed using the GeneGenious system and Gene Tool software (Syngene, Cambridge, UK).

2.6 Western Blotting

Cells were washed in 1xPBS and harvested in solution containing 150 mmol NaCl, 50 mmol Tris-HCl (pH 8.0), 0.1% sodium dodecyl sulfate SDS and the protease inhibitor cocktail COMPLETE (Roche-Boehringer Manheim, Roche Diagnostics, Lewes, East Sussex, UK). The lysates were spun at 13000 rpm for 10 min at 4°C and the supernatants were assayed for protein concentration using the BIO-RAD protein assay kit according to the manufacturer's instructions (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK). An equal amount of protein from each sample was boiled at 100°C in SDS sample buffer (100 mmol Tris-HCl, 4% SDS, 0.2% bromophenol blue and 20% glycerol) for 5 min and loaded onto a 15% SDS-polyacrylamide gel.

The proteins were transferred onto a polyvinyl membrane (Amersham Pharmacia Biotech, Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK) using the Trans-Blot SB Semi-Dry Transfer Cell apparatus (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK) according to the manufacturers specifications. After blocking for 20 minutes in 1xPBS, containing 5% non-Fat milk, 0.1% Tween 20 and 0.2% Sodium Azide (PBST), the membranes were blotted with the appropriate antibody (For a list of the various antibodies used and their dilutions see table 2.1) in PBST at 4°C overnight. After immuno-detection with the appropriate secondary antibody (table 2.1) (DAKO Ltd., Ely, Cambridgeshire, UK) diluted 1:2000 times in PBST for 2 hours the blots were

washed twice with PBST at room temperature and the blots were visualised using an enhanced ECL kit (Amersham Pharmacia Biotech, Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK).

After stripping in 0.2M NaOH for two washes of 5 min each and washing with PBST for 10 min the same blots were probed with goat anti-Actin-beta polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:2000 times in PBST at room temperature for 2 hours. Immunodetection and visualization was performed as described above. Densitometric analysis of western blots was performed in the linear range using a GS-800 Calibrated Densitometer (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK) linked to the Quantity One v 4.3 analysis software package (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK).

Table 2.1 List of antibodies used

Antibody	Type	Dilution for Immunoblotting	Dilution for Immunocytochemistry	Source
Anti-SAG	Rabbit poluclonal	1:1500	1:200	Gift by Sun Y.
Anti-Caspase-3	Mouse monoclonal	1:200	1:50	Novocastra, Newcastle, UK
Anti HOS	Goat polyclonal	N/A	1:100	Santa Cruz, USA
Anti-Actin	Goat polyclonal	1:2000	N/A	Santa Cruz, USA
Anti-MAPK p42/44	Rabbit polyclonal	1:1000	N/A	Cell Signalling
Anti-Akt	Rabbit polyclonal	1:1000	N/A	Cell Signalling
Anti-phospho-MAPK p42/44	Rabbit polyclonal	1:1000	N/A	Cell Signalling
Anti-phospho-Akt	Rabbit polyclonal	1:1000	N/A	Cell Signalling
Anti p38	Mouse polyclonal	1:1000	N/A	Cell Signalling
Anti-phospho-p38	Mouse polyclonal	1:1000	N/A	Cell Signalling
Anti-Kir 6.1	Mouse polyclonal	1:1000	N/A	Gift by Tinker A.
Anti-iPLA$_2$	Goat polyclonal	1:1000	N/A	Santa Cruz
Anti-PKCδ	Goat polyclonal	1:1000	N/A	Santa Cruz

Anti-PKCε	Goat polyclonal	1:1000	N/A	Santa Cruz
HRP conjugated Anti-Mouse IgG		1:2000	N/A	DAKO
HRP conjugated Anti Rabbit IgG		1:2000	N/A	DAKO
HRP conjugated Anti Goat IgG		1:2000	N/A	DAKO
Flurescent Anti mouse IgG		N/A	1:10000	ALEXA
Fluorescent anti-rabbit IgG		N/A	1:10000	ALEXA
Fluorescent anti-goat IgG		N/A	1:10000	ALEXA

2.7 Cloning

The wild type SAG gene and the mutant SAG MM14 were cloned in pcDNA 3.1 vectors (Gift from Sun Y.). For cloning the antisense SAG (GAS) plasmid, pcDNA 3.1-SAG plasmid was digested with XhoI and KpnI restriction enzymes (Promega UK Ltd, Southampton, UK) overnight at 37°C to release the SAG fragment which was subsequently purified by gel extraction and cloned in reverse orientation at the same sites on the pBluescript II KS- plasmid (Stratagene, La Jolla, CA, USA). Digestion with XhoI and SstI released the GAS fragment that was cloned in the corresponding sites of pcDNA3.1 (GIBCO BRL, Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA, USA).

Cells were co-transfected overnight with 2.5 μg of β-galactosidase plasmid (to mark the successfully transfected cells) and 2.5 μg of SAG, MM14 or pcDNA3.1. For transfection of GAS 1μg of the plasmid was transfected using LIPOFECTAMINE 2000 (GIBCO BRL, Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturers instructions. The day after, the cells were washed twice with 1xPBS and exposed to simulated ischaemia/reperfusion as described above. To discriminate between transfected and untransfected cells, after washing and fixing with 2.5% glutaraldehyde in PBS for 30 min at 25°C, the cells were stained with X-gal overnight at 37°C. Cells expressing the transfected β-galactosidase gene were stained blue.

2.8 Assessment of apoptosis.

The fixed cells were permeabilised with 0.1% Triton X-100 for 15 min and washed five times with PBS. Terminal deoxynucleotidyl transferase solution, containing 15 U/ml of terminal deoxynucleotidyl transferase (Roche-Boehringer Manheim, Roche Diagnostics, Lewes, East Sussex, UK) and 2.5 μM of fluorescein-conjugated dUTP (Roche-Boehringer Manheim, Roche Diagnostics, Lewes, East Sussex, UK), was added to the cells for 1.5 hours in a 37 °C humidified incubator. Cells were then washed five times with 1xPBS and 1% of propidium Iodide (w/v) was added to the cells for 2 min at 25°C. Following a triple wash in PBS cells were examined under an inverted fluorescent microscope. The percentage of apoptotic nuclei is expressed as a percentage of total nuclei from scoring at least 150 cells. Each count was repeated three times to make sure that the right number of cells per well was measured.

2.9 Statistics

Data are expressed as means ± S.D. Multiparametric analysis of variance (MANOVA) with a Bonferroni post-hoc test was performed for each group of treatments and significance was assumed when $P<0.05$. Differences among means were compared within the treatment groups using Student's *t* test and significance was assumed when $P<0.05$. The experiments were repeated at least three times. The SPSS Base 11.0 for Windows (SPSS Inc., Chicago, IL, USA)

statistical software package was used to perform all the statistical analyses described above

2.10 Peptides

Stresscopin (SCP) and stresscopin related peptide (SRP) were synthesized as described before (Hsu and Hsueh, 2001) and rat urocortin was purchased by Sigma (Sigma-Aldrich, Poole, UK). All peptides were diluted in 1XPBS and were used at a concentration of 10^{-8}M, unless otherwise stated.

2.11 Cardiomyocyte transfection

For assessing the effects of p42/44 MAPK, PKB/Akt and p38 MAPK on the cardioprotective effects of the peptides, primary neonatal cardiomyocytes were co-transfected overnight with 2.5μg of β-galactosidase plasmid (to mark the successfully transfected cells) and 2.5 μg of dominant negative MEK1 (activator of p42) (Lavoie et al., 1996), Akt (Kotani et al., 1999), MKK6 (an activator of p38) (Lavoie et al., 1996), or pcDNA3.1 (control), by the calcium phosphate method. The day after, the cells were washed twice with 1xPBS and exposed to simulated ischaemia/reperfusion as described. To discriminate between transfected and untransfected cells, after washing and fixing with 2.5% glutaraldehyde in PBS for 30min at 25°C, the cells were stained with X-gal overnight at 37°C. Cells expressing the transfected β-galactosidase gene were stained blue.

2.12 Hypoxic insult

Prior to induction of hypoxia cells were washed twice with phosphate buffered saline (1xPBS) and 1% FCS supported DMEM medium was added to the cells which were transferred to a gas chamber for induction of hypoxia in an atmosphere of 95% Argon and 5% CO_2 for the desired amount of time. After completion of the hypoxic treatment the cells were transferred to the incubator for up to16 hours in an atmosphere of 21% O_2 and 5% CO_2. Control cells were maintained in these conditions for the duration of the experiments.

2.14 Trypan Blue

For Trypan Blue exclusion assays, one million cells were harvested and spun down briefly by centrifugation at 900 rpm at room temperature for 1 min. The supernatant was discarded and cells were resuspended in 1xPBS. 50 µl of the cell suspension was added to 50 µl of 10% Trypan Blue (Sigma-Aldrich, Poole, UK), and 10µl were used to be counted on a Neubauer haematocytometric slide. Measurements were repeated at least three times for each experiment. At least, 700-900 cells were counted for each experiment.

2.15 Quantitative PCR

cDNA preparation and quantitative PCR

First strand synthesis was done using 1 μg of total RNA, and 1 μl of oligo-dT primers (Promega UK Ltd, Southampton, UK). AMV reverse transcriptase (Promega UK Ltd, Southampton, UK) was used according to the manufacturer's instructions. PCR was performed with the ABIprism lightcycler PCR to amplify the pool of cDNAs using QIAGEN SYBR Green system and the following primers

ANF forward: 5'-TGCCGGTAGAATGAGGTC-3'

ANF reverse: 5'-AGCCCTCAGTTTGCTTTTCA-3'

BNP forward: 5'-TAATCTGTCGCCGCTGGGAGGG-3'

BNP reverse: 5'-GAGCTGGGGAAAGAAGAGCCG-3'

GAPDH forward: 5'-GCCATCAACGACCCCTTCATTG-3'

GAPDH reverse: 5'-TGCCAGTGAGCTTCCCGTTC-3'

2.16 Measurement of Planimetric Area

Cells were observed under phase contrast or fluorescent light in the case of transfected cells, and the image was conveyed to a computer via a video camera. A mouse was used to draw around the perimeter of each cell and NIH image analysis was used to calculate the planimetric area. Measurements were calibrated by measurement of a known standard and results converted to μm.

2.17 Measurement of Protein:DNA ratio

Forty-eight hours after treatment cells were harvested by harvested by mechanical separation with a rubber policeman in ice cold 1xPBS and gently spun down and resuspended in ice cold 70% ethanol in PBS. Cells were fixed at 4° C overnight, centrifuged at 145xg for 2 min and resuspended in 100 µl FITC stain (0.1 µg/ml FITC, 50 µg/ml RNAse A in PBS) and were stained for 1-2 hours. Cells were then centrifuged as above and washed twice by resuspending in 1ml PBS. Cells were then stained with 400 µl of Propidium Iodide (PI) stain (50 µg/ml PI, 0.1% Na_3Citrate, 0.1% Triton X-100 in dH_2O). Cells were then analysed in a Coulter XL FACS maschine and mean fluorescence from 10000 cells at a time were measured for FITC (protein) at 525 nm and PI (DNA) at 620 nm. The mean ratio of protein to DNA was then calculated.

2.18 Blocking of signaling pathways

The LY294002 (Sigma-Aldrich, Poole, UK), a selective inhibitor of PI-3K, was used at a concentration of 50 µM. PD98059 (New England Biolabs Inc., Beverly, MA, USA), a selective inhibitor of MEK1/2 was used at a concentration of 100 µM, and SB203580 (Promega UK Ltd, Southampton, UK), a selective inhibitor of p38 MAPK was used at a concentration of 10 µM. The concentrations used are adequate to block the appropriate signaling pathways in rat neonatal cardiomyocytes, as described before (Lavoie et al., 1996; Brar et al., 2002; Brar et al., 2000). All inhibitors were used for 30 min prior to any other treatments.

CHAPTER III

Protective effects of the urocortin homologues against hypoxia/reoxygenation injury

3.1 Introduction

UCN has been shown to protect cardiac myocytes from cell death after exposure to hypoxia/reoxygenation injury *in vitro* (Brar et al., 1999; Brar et al., 2000). In addition, UCN has similar effects in the isolated perfused rat heart (Brar et al., 2000). As CRFR2β is the only subtype expressed in the rat heart (Nishikimi et al., 2000), the cardioprotective effects of UCN against hypoxia/reoxygenation (Okosi et al., 1998; Brar et al., 1999; Brar et al., 2000; Latchman, 2001; Brar et al., 2002; Latchman, 2002) are attributed to the binding to the CRRFR2. Since SCP and SRP show higher affinity for the CRFR2 than UCN (Hsu and Hsueh, 2001; Lewis et al., 2001), we hypothesised that these peptides might exhibit higher protective effects than UCN and, or might produce these effects with higher specificity, since they bind only to CRFR2.

3.2 Results

3.2.1 UCN Homologues are expressed in Rat primary cardiomyocytes

Initially, we wished to determine whether the UCN homologous genes SCP and SRP are expressed in neonatal rat cardiomyocytes. We isolated total mRNA from untreated cardiomyocytes (Lanes 1, 2, 1' and 2', Fig. 3.1) and cardiomyocytes that were exposed to 4 hours of hypoxia, followed by 16 hour reoxygenation (Lanes 3 and 4 and 3' and 4', Fig. 3.1). We failed to amplify SRP in the untreated cardiomyocytes (lane 2, Fig.3.1), although SRP was expressed in the cardiomyocytes after hypoxia/reoxygenation injury (lane 4, Fig. 3.1). SCP was expressed in untreated cardiomyocytes and after hypoxia/reoxygenation (Fig. 3.1). SRP was present at a similar level to SCP after hypoxia/reoxygenation, although it was undetectable prior to the insult (Fig. 3.1).

In addition, we tried to confirm the above results by performing real time PCR on the above samples, as it was previously suggested that SRP is actually expressed in the heart (Hsu and Hsueh, 2001). This time, we managed to detect SRP mRNA in normoxic conditions, albeit the levels of SRP were 5 times lower than SCP mRNA levels (Fig. 3.2). After hypoxia reoxygenation the SCP and SRP levels were increased to similar levels and were comparative to the levels detected by our quantitative PCR methods (Fig. 3.1 and 3.2)

These results show that SCP and SRP are expressed in the rat neonatal cardiomyocytes and that their levels are increased following a hypoxic insult.

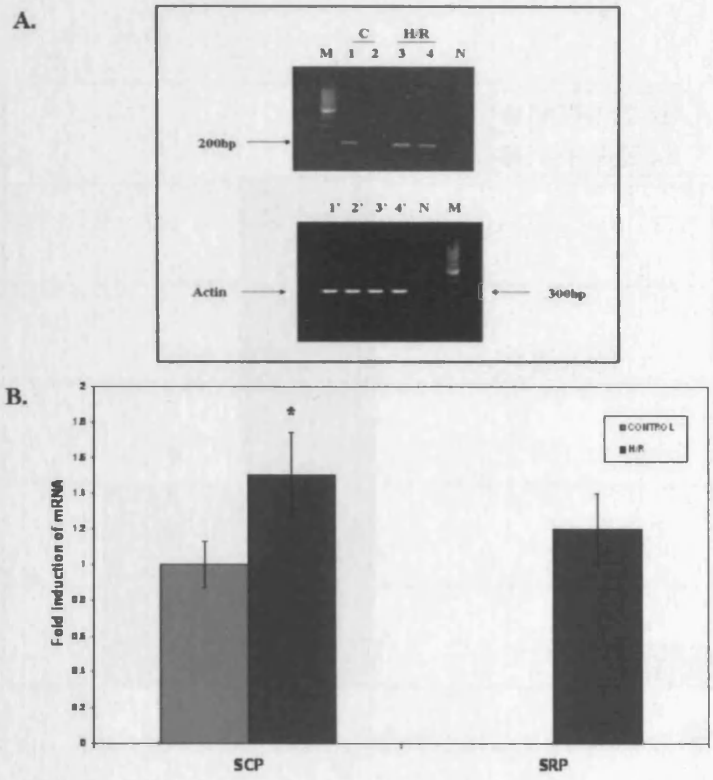


Figure 3.1. SCP and SRP mRNA are induced by hypoxia/reoxygenation. **A.** Ethidium Bromide stained agarose gel of RT-PCR amplification products for SCP (lanes 1 and 3) and SRP (lanes 2 and 4) and actin (lanes 1'-4') from RNA isolated from untreated rat neonatal cardiomyocytes (lanes 1, 2 and 1',2') and from rat neonatal cardiomyocytes undergone Hypoxia/Reoxygenation (lanes 3, 4, 3' and 4'). Lane M is a 1kb DNA marker and lanes N are negative controls. **B.** Densitometric analysis SCP and SRP RT-PCRs. Actin was used for normalisation. Values are the average of at least three experiments with their standard deviation represented by the bars. Stars represent statistical significance (2-way ANOVA, $p<0.05$).

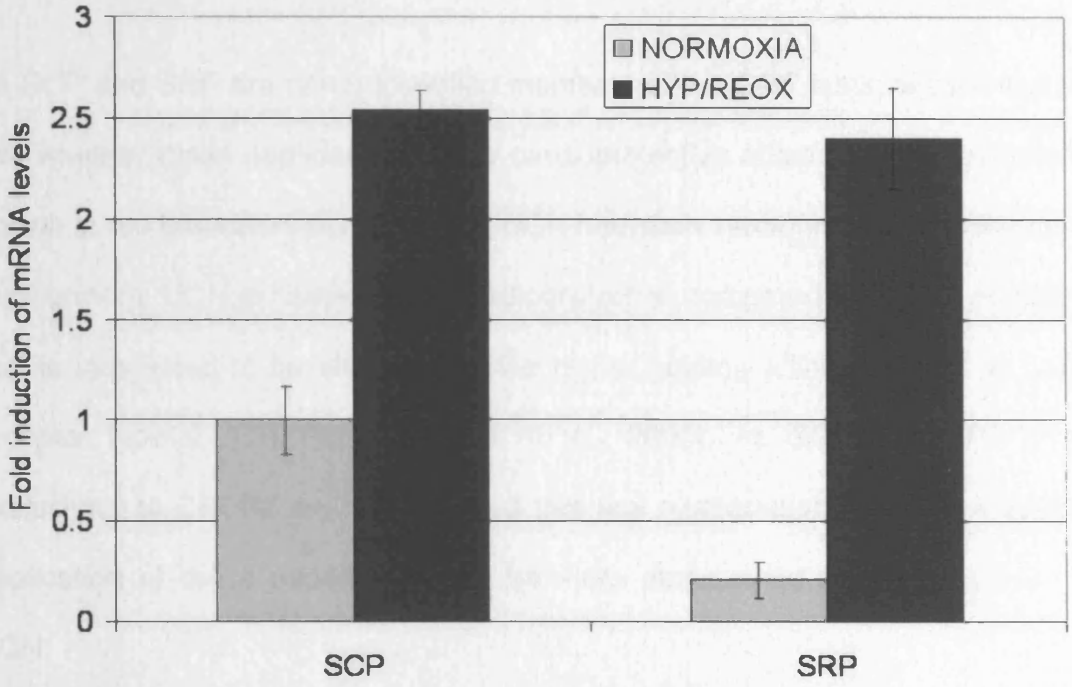


Figure 3.2. Quantification of SCP and SRP mRNA levels in primary rat neonatal cardiomycytes by real time PCR. Columns represent the mean fold change over control of four experiment and bars are SEM.

3.2.2 Protective properties of UCN homologues

As SCP and SRP are newly identified members of the CRF family we wanted to test whether these peptides have any cardioprotective effects. It was previously shown in our laboratory that CRF and UCN had such cardioprotective properties. Furthermore, UCN exhibited better cardioprotection compared to CRF, a property that is suggested to be attributed to the higher binding affinity of UCN to CRF receptor type 2 (CRFR2) (Nishikimi et al., 2000). As SCP and SRP bind exclusively to CRFR2 we hypothesised that any cardioprotection elicited by the application of these peptides should be more pronounced when compared to UCN.

To test cardioprotective properties of the UCN-related peptides we incubated primary cardiomyocytes with 1 nM of each peptide for 1 hour prior to subjecting the cells to an anoxic environment for 4 hr. After a 16 hr reoxygenation, the cells were harvested and stained with Trypan Blue. In these experiments, some death was observed on untreated cells, due to damage during the isolation and harvesting procedures. However, a dramatic increase in cell death was observed upon exposure of the cells to hypoxia/reoxygenation.

As shown in Fig. 3.3 all peptides improved the viability of the primary cardiomyocytes exposed to hypoxia/reoxygenation (2-way ANOVA, $p<0.05$ vs

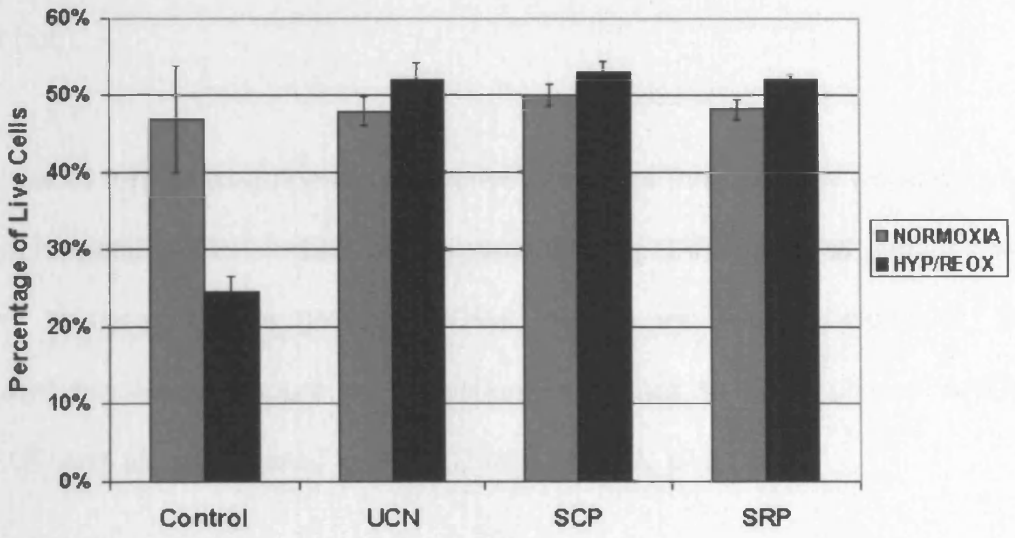


Figure 3.3. UCN and its homologues protect cardiomyocytes from cell death induced by hypoxia/reoxygenation. Percentage of live cells as assessed by trypan blue exclusion is represented by the columns. Values are the average of at least three experiments with their standard deviation represented by the bars. For statistical differences see text.

Control). We could not detect any difference in the levels of protection among the three different peptides at this concentration. (2-way ANOVA, $p>0.05$, Bonferroni post-hoc).

In order to further examine the protective effects of the peptides we added 1nM of each peptide at the onset of reoxygenation (Fig. 3.4). Again all peptides were found to be protective (2-way ANOVA, $p<0.05$ vs Control). However, SRP showed the least amount of protection compared to SCP (2-way ANOVA, $p<0.05$) and also compared to UCN (2-way ANOVA, $p<0.05$).

When we compared the levels of protection of the different peptides when added prior to, or following hypoxia, we observed that the levels of protection for UCN and SCP are no different, regardless of when we added the peptides (2-way ANOVA, $p>0.05$ followed by Bonferroni post-hoc between SCP at reoxygenation vs SCP at hypoxia and $p>0.05$ between UCN at reoxygenation vs UCN at hypoxia). SRP in comparison showed higher levels of protection when added prior to hypoxia (2-way ANOVA Bonferroni post-hoc, $p<0.05$ between SRP at hypoxia vs SRP at reoxygenation).

The above results indicate a slight difference in the levels of protection among the different peptides. Moreover, it shows that SCP, like UCN, is able to attenuate cell death, even if it is administered after the hypoxic insult.

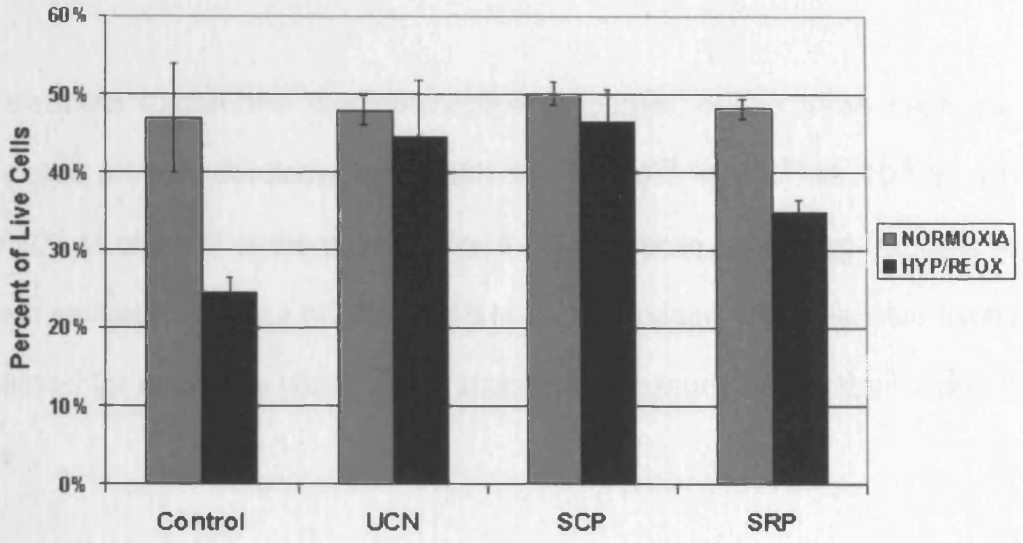


Figure 3.4. UCN and its homologues can protect cardiomyocytes from cell death caused by hypoxia/reoxygenation when added at the onset of reoxygenation. Percentage of live cells as assessed by trypan blue exclusion is represented by the columns. Values are the average of at least three experiments with their standard deviation represented by the bars.

3.2.3 Antiapoptotic properties of UCN homologues

To examine further the cardioprotective properties of the three peptides we incubated primary cardiomyocytes with 10^{-12}M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M and 10^{-7} M of each of the peptides for 1 hour, prior to subjecting the cells to an anoxic environment for 4 hr. After a 16 hr reoxygenation, the cells were fixed and assessed for apoptosis with TUNEL staining to measure the number of apoptotic cells.

All three peptides were strongly protective at all concentrations used compared to the control (2-way ANOVA Bonferroni post-hoc, $p<0.05$ vs control) (Fig. 3.5). When we compare the protective effects between the peptides, SCP exhibited higher levels of protection from apoptosis compared to UCN at all concentrations up to and including10^{-10} M (2-way ANOVA Bonferroni post-hoc, $p<0.05$) (Fig. 3.5). Similarly, SRP was more protective than UCN for concentrations up to 10^{-10} M (2-way ANOVA Bonferroni post-hoc, $p>0.05$). However, at a concentration of 10^{-9} M UCN was the more protective of the two (Fig. 3.5) (2-way ANOVA Bonferroni post-hoc, $p>0.05$). SCP was even more protective than SRP when used at concentrations of 10^{-12} M and 10^{-9} M. For concentrations between 10^{-11} M and 10^{-8} M the two peptides exhibited similar cardioprotective effects (2-way ANOVA Bonferroni post-hoc, $p<0.05$) (Fig. 3.5).

When the peptides were added at the onset of reoxygenation, again they all showed antiapoptotic properties at all concentrations examined (2-way ANOVA Bonferroni post-hoc, $p<0.05$ vs control) (Fig. 3.6). Again, SCP was significantly more protective than UCN at concentrations of 10^{-12} and 10^{-8} M (2-way ANOVA Bonferroni post-hoc, $p<0.05$) and there was no difference in the levels of protection between SCP and SRP for any of the concentrations used (2-way ANOVA Bonferroni post-hoc, $p>0.05$). SRP was more protective than UCN only at concentration of 10^{-8} M (2-way ANOVA Bonferroni post-hoc, $p<0.05$).

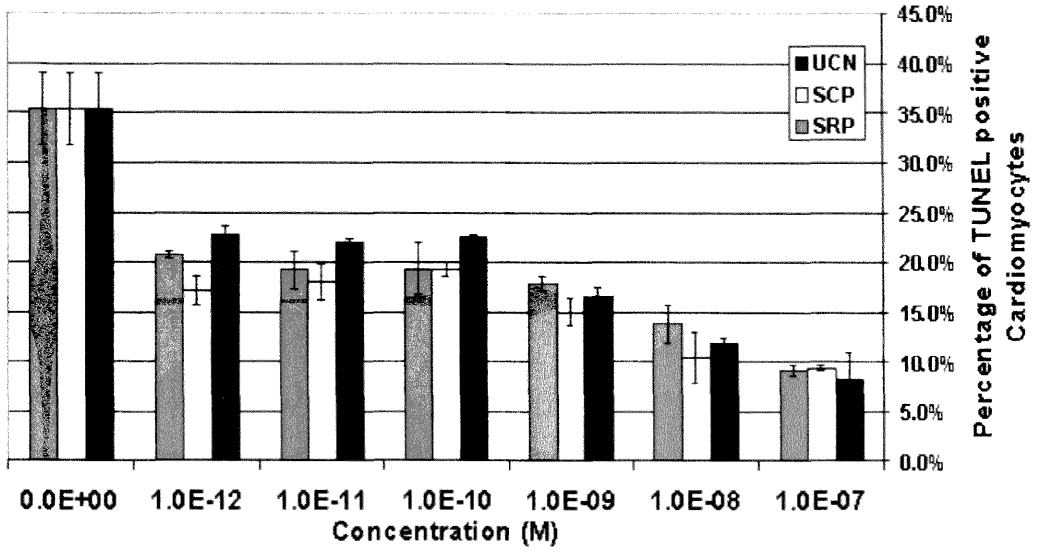


Figure 3.5. UCN and its homologues can protect cardiomyocytes from apoptotic cell death caused by hypoxia/reoxygenation. Percentage of TUNEL positive cardiomyocytes is represented by the columns. Values are the average of at least three experiments with their standard deviation represented by the bars.

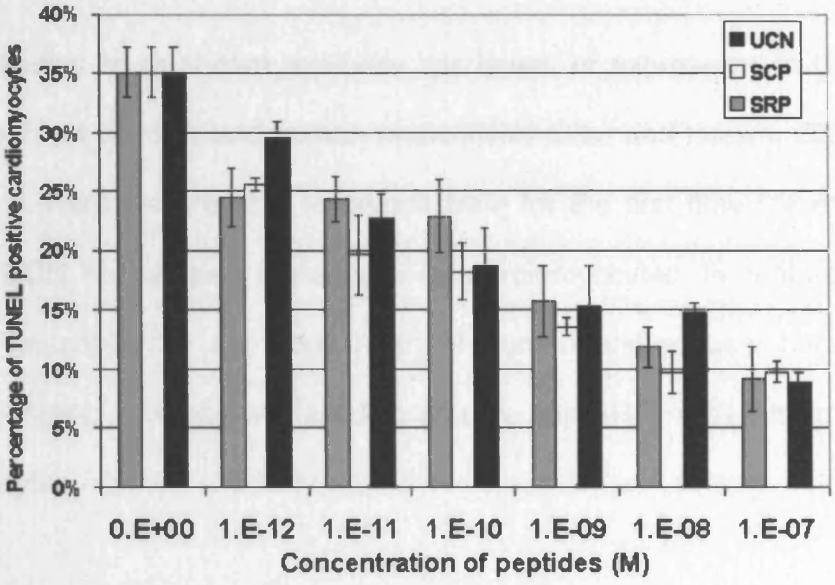


Figure 3.6. UCN and its homologues can protect cardiomyocytes from apoptotic cell death caused by hypoxia/reoxygenation when added at the onset of reoxygenation. Percentage of TUNEL positive cardiomyocytes is represented by the columns. Values are the average of at least three experiments with their standard deviation represented by the bars.

3.2.4 Discussion

Previous studies have shown relatively low levels of expression of UCN III or SCP in the heart of mice and human respectively (Hsu and Hsueh, 2001; Lewis et al., 2001). Here we are able to demonstrate for the first time the expression pattern of UCN homologues in neonatal rat cardiomyocytes. In contrast to what has been described for the adult heart of human and mouse, here SCP is expressed highly, whereas we failed to amplify any SRP mRNA from neonatal cardiomyocytes.

However, again for the first time we are able to show that the mRNAs encoding both peptides are induced after a hypoxia/reperfusion injury (Fig. 3.1). We have not examined the expression levels of the peptides in the intact heart or in adult rat cardiomyocytes. Furthermore, although we were unable to detect any SRP mRNA with conventional PCR methods, we were able to show that SRP is indeed expressed in the neonatal cardiomyocytes, albeit in lower levels than SCP by real time PCR (Fig. 3.2). These results do demonstrate the higher sensitivity of the real time PCR method. It is possible that SRP might become the predominant peptide in adult life.

Urocortin has been shown to be able to bind to CRFR2 types with higher affinity than to CRFR1 and binds with higher affinity to CRFR2 than CRF (Vaughan et al., 1995). In addition, the exclusive presence of CRFR2β in the heart (Nishikimi et al., 2000) and the fact that UCN failed to increase cardiac function in CRFR2

knock out mice (Coste et al., 2000) has always led to the speculation that CRFR2 is responsible for the effects of UCN in the periphery. As a result, we initially hypothesised that because of the higher affinity of the SCP and SRP peptides for the CRFR2β (Hsu and Hsueh, 2001; Lewis et al., 2001) these peptides might exhibit augmented cardioprotective properties in the heart compared to UCN. In this study we are able to show that all three homologues exhibit similar cardioprotective effects. Nevertheless, SCP and SRP were able to promote higher levels of protection against apoptosis when 1pM of the peptides were used (Fig. 3.5 and 3.6). Hence, cardioprotective effects can be observed with UCN-related peptides which bind only to the CRFR2.

UCN II and III have been recently shown to exhibit potent cardioprotective effects against a hypoxic insult in neonatal cardiomyocytes, (Brar et al., 2004a) in adult cardiomyocytes and in Langerdorff perfused hearts (Brar et al., 2004a). The effects of the two peptides was comparable or higher than UCN, and most importantly the administration of the peptides in the presence of a specific CRFR2 inhibitor, or in CRFR2-/- mice failed to exhibit any cardioprotection, showing that UCN II and III bind exclusively to CRFR2 to elicit their cardioprotection (Brar et al., 2004a).

It is worth noting that although SRP has a higher affinity for the CRFR2β compared to SCP (Lewis et al., 2001), in our studies SCP usually showed higher protective effects compared to SRP (Fig. 3.4, 3.5 and 3.6). This could be due to a

possible lower affinity of the human SRP to the rat receptor or because of the higher ability of SCP to activate the receptor CRFR2β compared to UCNII/SRP, as measured by accumulation of cAMP (Lewis et al., 2001). This could also explain the similar protective properties of UCN to the two homologues, as the relative potencies of SCP, SRP and rat UCN to functionally activate the receptor, overlap (Lewis et al., 2001).

In summary, we showed for the first time that SCP and SRP are expressed in the neonatal cardiomyocytes. We also demonstrated that they are peptides with higher or equal cardioprotective properties than UCN, but more importantly they are more specific than UCN as they do not bind to CRFR1, making them better candidates as possible therapeutic agents.

CHAPTER IV

Hypertrophic effects of the UCN homologues

4.1 Introduction

UCN not only is able to induce cardioprotective effects in cardiac myocytes and the whole heart, but has also been shown to be involved in cardiac hypertrophy. UCN is able to increase distinct markers of hypertrophy, such as protein synthesis, cell size, ANP and BNP natriuretic peptide secretion and collagen in cardiomyocytes (Ikeda et al., 1998; Nishikimi et al., 2000; Railson et al., 2002). In addition, expression of UCN mRNA was higher in human hearts with left ventricular hypertrophy (Nishikimi et al., 2000) and UCN immunoreactivity was more intense in cardiomyocytes of human failing hearts, than in normal human hearts (Nishikimi et al., 2000).

In our current study, we compare the hypertrophic properties of UCN, SCP and SRP in rat neonatal cardiomyocytes. As SCP and SRP are more specific for CRFR2 than UCN it would be interesting to examine whether any differences in the hypertrophic effects of the three peptides do occur.

4.2 Results

4.2.1 UCN Homologues increase cell size

UCN has been previously shown to increase cardiomyocyte size (Railson et al., 2002). As SCP and SRP have a higher affinity for the CRFR2 than UCN and they do not bind to CRFR1, we wanted to examine whether their administration to primary cardiomyocytes would have a similar effect to UCN on cell size. We chose to use the measurement of the cell area as an indicator of cell size. When 10^{-8} M of each peptide was added to the cells for 48 hours a distinct increase in cell area was observed (MANOVA $p<0.05$) (Fig. 4.1A). Furthermore, SCP was the most potent of the three peptides in increasing cell area, whilst SRP and UCN had similar effects on cell area (MANOVA $p>0.05$) (Fig. 4.1A). UCN and SRP caused an approximately 15% increase in cell area over control, whereas SCP caused an approximately 35% increase in cell area comparable to the increase caused by phenylephrine, a known hypertrophic factor (MANOVA $p>0.05$).

These results therefore, show that all three peptides are causing an increase in cell size and that SCP is the most potent of the three at 10^{-8} M concentration.

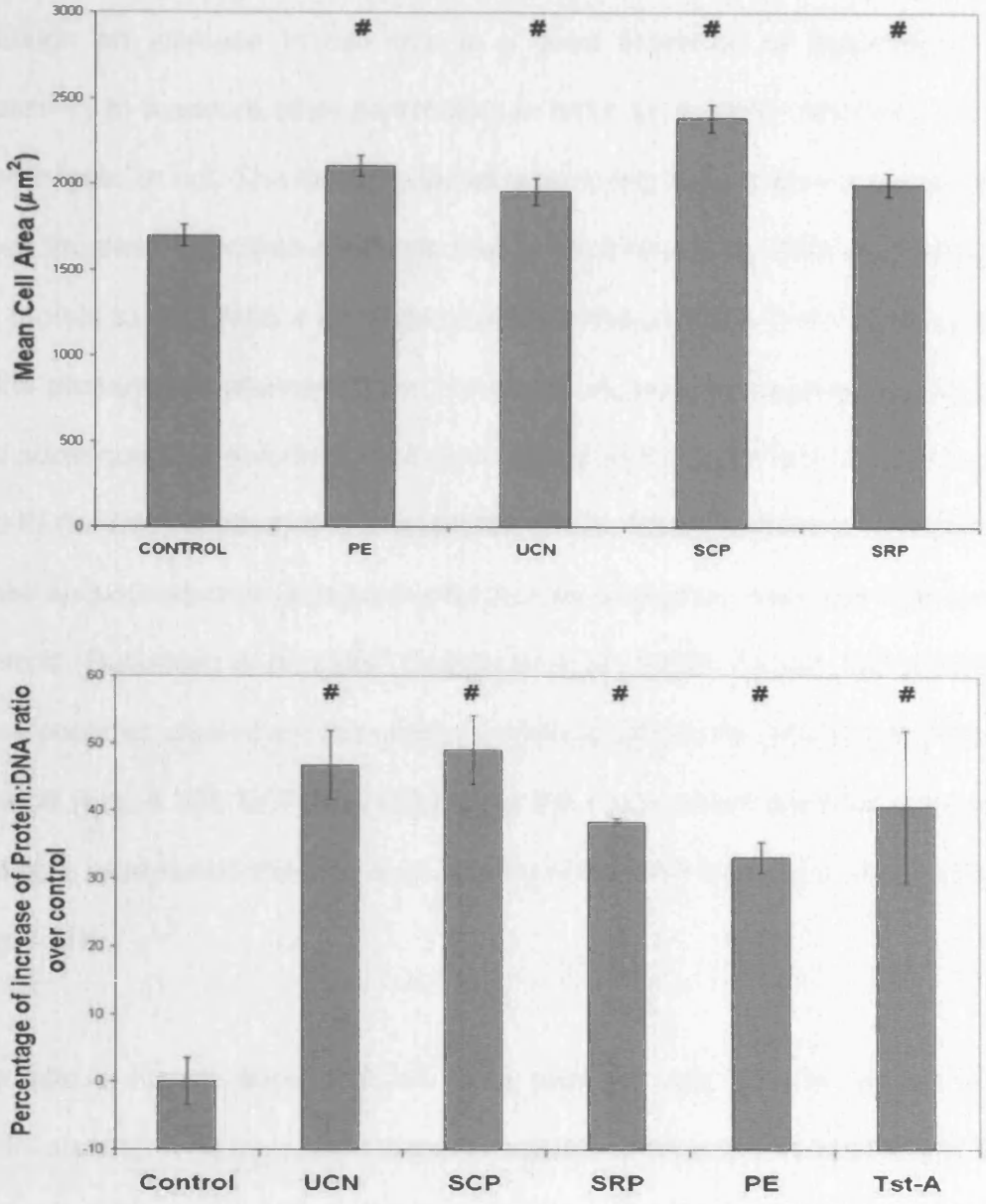


Figure 4.1. (A) Effects of the UCN peptides on cell size. 10^{-8} M of each peptide was added to the medium of rat neonatal cardiomyocytes for 48 hours, prior to measuring their cell area. Columns represent the mean of four experiments and bars are SEM. # represent $p<0.05$ after MANOVA test. (B). Effect of the UCN peptides on protein:DNA ratio. 10^{-8} M of each peptide was added to the medium of rat neonatal cardiomyocytes for 48 hours, prior to measuring their protein:DNA ratio by FACS. Columns represent the mean change in percentage over control of six experiments and bars are SEM. # represent $p<0.05$ after MANOVA test.

4.2.2 UCN homologues induce protein synthesis

Although an increase in cell size is a good indication of hypertrophy, it is necessary to measure other parameters in order to establish whether a factor is hypertrophic or not. The increase in cell size during hypertrophy is accompanied by an increase in protein synthesis that is not followed by DNA replication. So, the protein to DNA ratio is a reliable marker of hypertrophy. Cells were incubated in the presence of phenylephrine, trichostatin A, and the peptides for 48 hours and subsequently harvested, fixed and stained by FITC (for total protein content) and PI (for DNA content) and analysed by FACS. Phenylephrine and Trichostatin A are known inducers of hypertrophy and as such they were used as positive controls (Gusterson et al., 2002; Gusterson et al., 2003). As with the cell size, all three peptides caused an increase in protein to DNA ratio (MANOVA, $p<0.05$ to control) (Fig. 4.1B). SCP and UCN were the more potent peptides with a 49% and 46% increase on the ratio over control, whilst SRP induced a 38.5% increase (Fig. 4.1B).

The above results show that all three peptides are able to induce protein synthesis after a 48 hour administration and their effects are comparable to those of known hypertrophic agents like phenylephrine and Trichostatin A.

4.2.3 UCN homologues induce the expression of Atrial Natriuretic and Brain Natriuretic peptides

Hypertrophy is characterised by a switch to a fetal gene program. The ANP and BNP genes fall into this category and their expression is shown to be elevated in patients with progressive heart failure. CRF and UCN has been previously shown to induce expression of ANP and BNP (Grunt et al., 1992; Haug et al., 1994; Tojo et al., 1996; Ikeda et al., 1998). To examine the effects of SCP and SRP on ANP and BNP expression we treated rat cardiomyocytes for 48 hours and analysed the mRNA levels in the cells using quantitative real time PCR analysis. All three peptides were able to induce the expression of ANP and BNP mRNA after 48 hours (MANOVA, $p<0.05$ vs control) (Fig. 4.2). SCP caused a 2.5 fold induction of ANP mRNA and a 5.5 fold induction of BNP (Fig. 4.2). UCN was more potent than SRP in inducing the natriuretic peptides, but less pronounced in the induction of ANP compared to SCP (MANOVA, $p<0.05$). In comparison, all three peptides are able to induce BNP levels more efficiently than ANP.

In conclusion, all three peptides are able to cause an increase in all the hypertrophic markers we examined and SCP appears to be the most potent of three peptides, exactly as shown before for its cardioprotective effects compared to UCN and SRP (Chapter III).

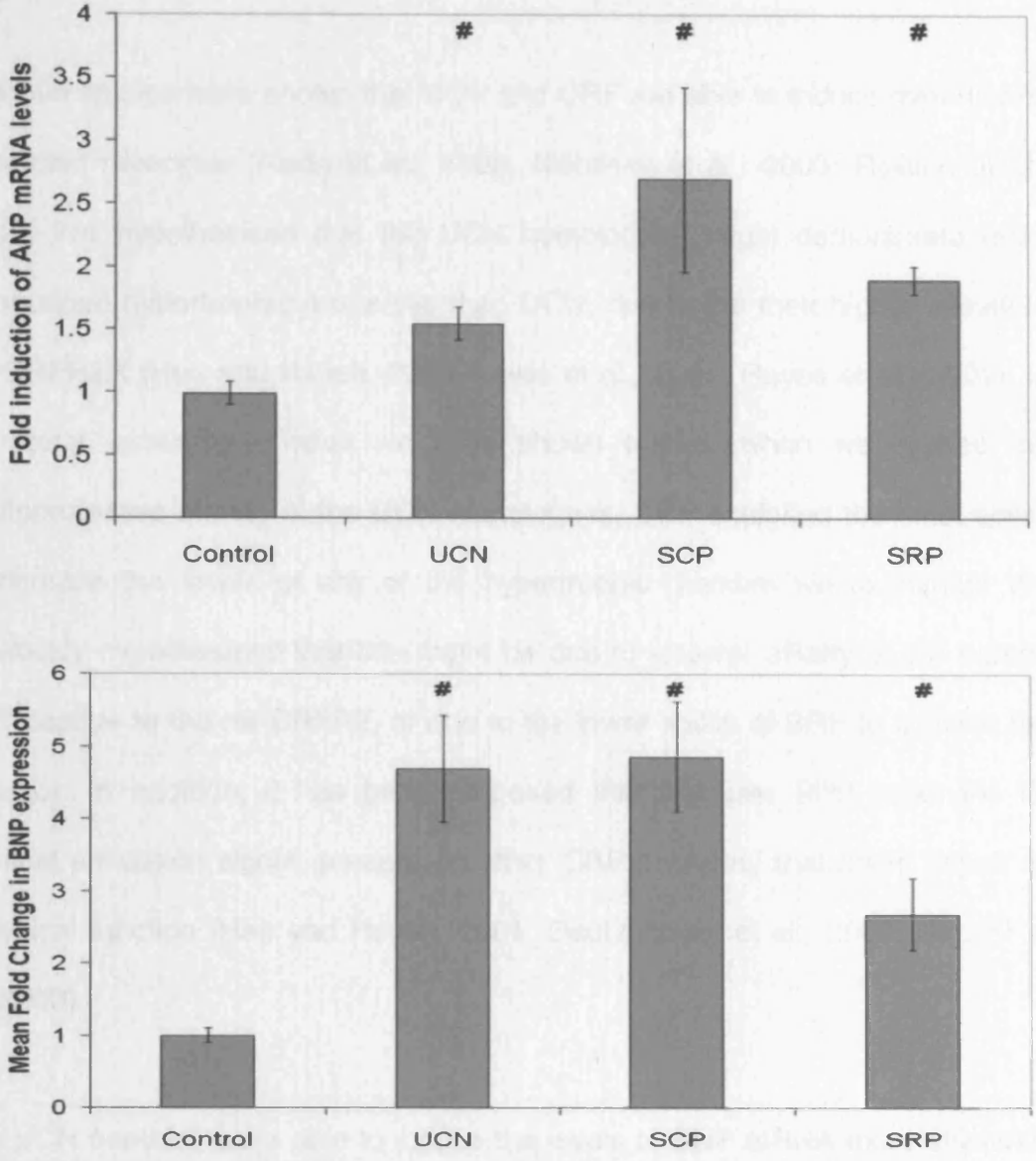


Figure 4.2. Effects of the UCN peptides on the expression of the genes encoding (A) Atrial natriuretic peptide and (B) Brain natriuretic peptide. 10^{-8} M of each peptide was added to the medium of rat neonatal cardiomyocytes for 48 hours, prior to measuring the changes on gene expression by real time PCR. Columns represent the mean fold change over control of four experiment and bars are SEM. # represent $p<0.05$ after MANOVA test.

4.3 Discussion

Previous studies have shown that UCN and CRF are able to induce hypertrophy in cardiac myocytes (Ikeda et al., 1998; Nishikimi et al., 2000; Railson et al., 2002). We hypothesised that the UCN homologues might demonstrate more pronounced hypertrophic properties than UCN, due to the their higher affinity to the CRFR2R (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). In our study, similarly to what we have shown before, when we studied the cardioprotective effects of the UCN homologues, SRP exhibited the least ability to increase the levels of any of the hypertrophic markers we examined. We previously hypothesised that this might be due to a lower affinity of the human SRP peptide to the rat CRFR2, or due to the lower ability of SRP to activate the receptor. In addition, it has been proposed that because SRP lacks the C-terminal amidation signal, present on other CRF peptides, that might impair its biological function (Hsu and Hsueh, 2001; Dautzenberg et al., 2002; Hauger et al., 2003).

The UCN peptides were able to induce the levels of BNP mRNA more efficiently than ANP (Fig. 5). This is in agreement with previous results where, although, the levels of ANP protein were higher than BNP in rat neonatal cardiomyocytes, the fold induction of BNP protein levels were higher than ANP after UCN administration (Ikeda et al., 1998). This difference between the two natriuretic peptides would indicate some difference in the gene regulation. It could be that a

factor or factors that are induced by UCN administration might have a different affinity for the two gene promoters, or that one promoter is exclusive for a certain factor or factors. Clearly closer examination and studies into the promoter region of the two genes could provide us with some clues concerning the promoter regulating peptides that are induced by UCN and are responsible for the hypertrophic effects.

In conclusion, it appears that all three peptides are able to protect cardiac cells from hypoxia/reoxygenation injury, but unfortunately at the same time they do induce hypertrophy. If we were able to find some differences among the mechanisms that induce cardioprotection and hypertrophy we might be able to isolate those that cause hypertrophy, in order to use the peptides as protective agents. For these reason we are going to examine the pathways that UCN and its homologues are inducing.

CHAPTER V

Signalling Pathways involved in protective and Hypertrophic Effects of UCN homologues

5.1 Introduction

Cardioprotection from hypoxia/reoxygenation injury induced by UCN and its homologues in rat neonatal cardiomyocytes is dependent on activation both of Akt and MAPK p42/44, as inhibiton of Akt and p42/44 pathways by chemical inhibitors, or with overexpression of dominant negative constructs abolished cardioprotection by the peptides (Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al., 2002b; Railson et al., 2002; Schulman et al., 2002; Brar et al., 2004a; Brar et al., 2004b). In addition, the level of activation of MAPK p42/44 phosphorylation is correlated to the cardioprotective effects of the peptides, as UCN II and III are able to induce higher phosphorylation levels of MAPK p42/44 than UCN (Brar et al., 2004a; Brar et al., 2004b). Furthermore, it was shown that the MAPK p42/44 pathway is not involved in the manifestation of the hypertrophic effects of UCN in rat neonatal cardiomyocytes, as transfection of cardiomyocytes with a dominant negative MEK1 construct, prior to UCN administration, failed to attenuate the increase in mean cell area by UCN (Railson et al., 2002).

It was previously postulated that the increase in cAMP by UCN administration is mediating its hypertrophic effects (Nishikimi et al., 2000; Rademaker et al.,

2002). However, Akt activation has also been implicated in cardiac hypertrophy (Frey and Olson, 2003), but its role in the hypertrophic effects of UCN or its homologues has not been studied. In this chapter we will try to establish the role of MAPK and Akt in the cardioprotective and hypertrophic effects of UCN and its homologues.

5.2 Results

5.2.1 UCN homologues activate MAPK and Akt

We wanted to establish whether the differences in cardioprotection that we observed between the three peptides were due to differences in their ability to activate certain signalling molecules, such as p42/44 MAPK, and Akt.

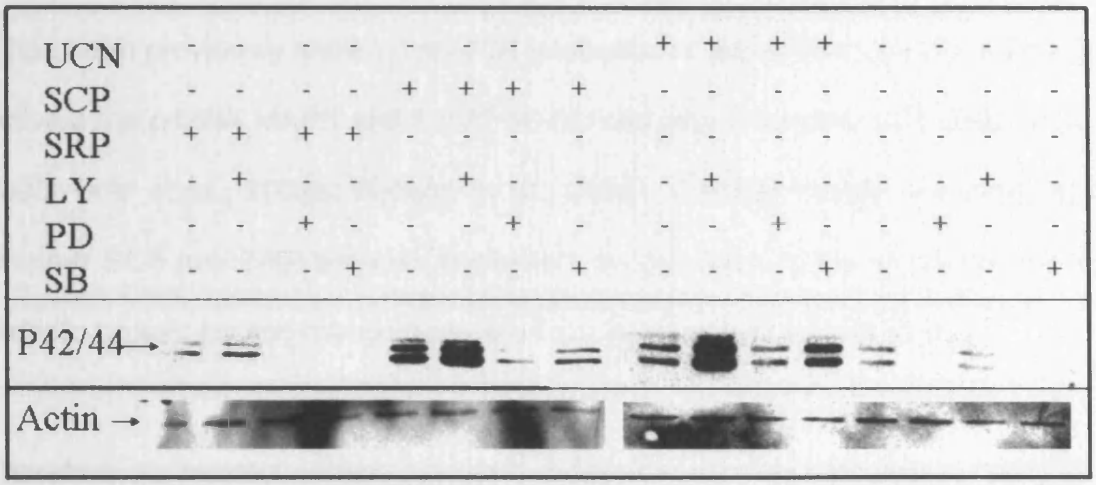
To do this, we treated cardiomyocytes for 30 minutes with the p42/44 MAPK inhibitor PD98059 prior to the addition of 10^{-8} M of UCN, SCP or SRP. Also, we treated cells with the peptides alone to compare the levels of phosphorylated p42/44 MAPK between the different sets of cells. After one hour of incubation in the presence of one of the three peptides and the presence or absence of the inhibitor, the cells were harvested and analysed by western blotting with an antibody specific for phosphorylated p42/44 MAPK.

SCP treatment leads to a more pronounced p42/44 MAPK phosphorylation than UCN and UCN exhibits a higher phosphorylation signal than SRP (Fig. 5.1). The ability of the peptides to activate the p42/44 MAPK pathway seems to correspond with their cardioprotective potency, as SCP appears to be slightly more potent than UCN and SRP at least at lower concentrations. The presence of the inhibitor PD203580 completely abolished p42/44 MAPK phosphorylation by the peptides. So, the ability of the peptides to protect is connected with their ability to activate the p42/44 MAPK signalling pathway in rat neonatal cardiomyocytes.

We examined the levels of phosphorylation of Akt induced by the three peptides, in order to discover if the UCN homologues are able to induce phosphorylation of Akt. To do this we treated cardiomyocytes for 30 min prior to the peptide addition with the PI3-K inhibitor LY294002. Also cells were treated with the peptides alone to compare the effects of the inhibitor on the Akt phosphorylation. After one hour of incubation in the presence of one of the three peptides and the presence or absence of the inhibitor, the cells were harvested and analysed by western blotting with an antibody specific for phosphorylated Akt.

SCP was able to induce more pronounced phosphorylation of Akt, than UCN and SRP (Fig. 5.1). Furthermore, LY294002 was able to block Akt phosphorylation in the concentration used. Interestingly, blocking the MAPK pathway caused an increase on the levels of phosphorylation of Akt (Fig. 5.1).

Although, we were aware that p38 MAPK is not activated after CRFR2 activation by either CRF or UCN we treated neonatal rat cardiomyocytes with the p38 MAPK inhibitor SB203580, to examine any possible effects of p38 MAPK inhibition on the levels of p42/44 MAPK and Akt activation (Fig. 5.1). Interestingly, blockage of the p38 MAPK pathway with SB203580 enhanced the ability of SCP to activate p42/44 MAPK. In contrast, blockage of the Akt pathway with LY294002 enhanced the ability of UCN and SCP to activate p42/44 MAPK (Fig. 5.1).



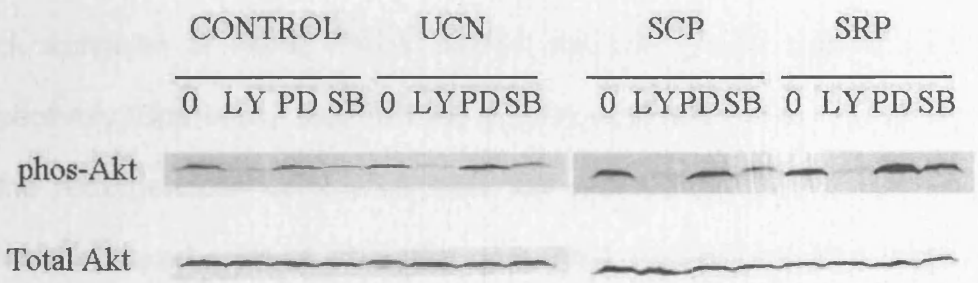


Figure 5.1. Phosphorylation of p42/44 MAPK and Akt by UCN SCP and SRP on rat neonatal cardiomyocytes. Western blotting analysis of phosphorylated p42/44 MAPK (Top) and phosphorylated Akt (Bottom) by the UCN peptides in the presence and absence of specific kinase inhibitors. LY: LY294002 a PI3-K inhibitor, PD: PD98509 a p42/44 MAPK inhibitor, SB: SB203580 a p38 MAPK inhibitor.

5.2.2 MAPK activation and Akt activation is required for cardioprotective effects of UCN homologues.

It has been previously shown that UCN protection is dependent upon its ability to activate the p42/44 MAPK and the PI-3K/Akt pathway in cardiac cells (Brar et al., 2000; Brar et al., 2002a; Railson et al., 2002). For this reason we examined whether SCP and SRP are also dependent on activation of the same kinases in order to protect primary cardiomyocytes from hypoxia/reperfusion injury.

Therefore we treated primary rat cardiomyocytes with the inhibitors LY294002, PD98059 and SB203580 for 30 minutes prior to the addition of 10^{-8}M UCN, SCP and SRP and following hypoxia reoxygenation. The above inhibitors are known to block activation of PI3-K, MAPK p42/44 and p38 kinase signalling pathways, respectively (Brar et al., 2000; Brar et al., 2002a; Railson et al., 2002). At the end of the reoxygenation cells were fixed and stained by TUNEL, a method that stains the nicked ends of DNA in a cell with a fluorescent uridyl nucleotide, to reveal DNA that has been cut with CAD, as a result of apoptosis taking place. This assay will also reveal the antiapoptotic effects of UCN and its homologues and will strengthen the results we obtained by the use of the Trypan Blue assay.

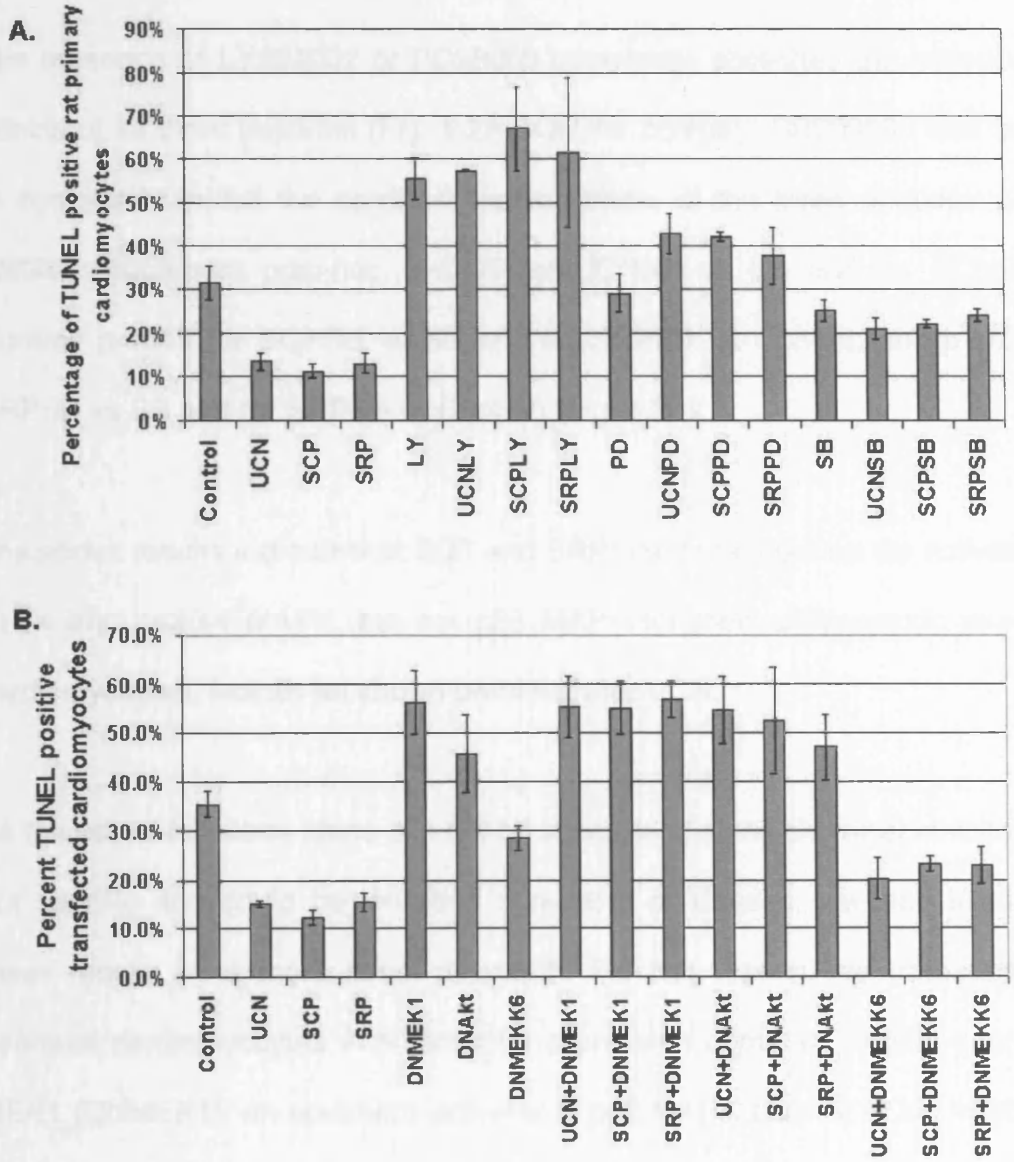


Figure 5.2. The antiapoptotic properties of UCN and its homologues are attenuated by blocking the activation of p42/44 MAPK and PI-3K. **A.** The effect of chemical inhibitors on the cardioprotective effects of UCN and its homologues. PD98059 was used to block p42/44 activation, LY294002 was used to block PI-3K activation and SB203580 for the blocking of p38 MAPK. The inhibitors were used as described in the Materials and Methods. **B.** Abrogation of the activation of p42 and Akt attenuates the cardioprotective effects of UCN and its homologues. Cells were transfected with DNMEK1 to block p42/44 activation, DNAkt to block PI-3K activation and DNMEKK6 to block p38 MAPK, prior to undergoing simulated ischaemia/reoxygenation injury. The transfections were carried out as described in the Materials and Methods.

The presence of LY294002 or PD98059 completely abolished the antiapoptotic effects of all three peptides (Fig. 5.2A). On the contrary, SB203580 was unable to completely inhibit the cardioprotective effects of the three peptides (2-way ANOVA Bonferroni post-hoc, $p<0.05$ for UCNSB vs SB and for UCNSB vs Control; $p<0.05$ for SCPSB vs SB and for SCPSB vs Control; and $p<0.05$ for SRPSB vs SB and for SRPSB vs Control) (Fig. 5.2A).

The above results indicate that SCP and SRP are dependent on the activation of PI-3K and p42/44 MAPK, but not p38 MAPK for their antiapoptotic effects in cardiomyocytes, exactly as shown previously for UCN.

As the use of inhibitors alone can not be conclusive, since chemical inhibitors are not specific and could be inhibiting a number of kinases, we tried to support these results by a more direct approach. For this reason, we transfected rat neonatal cardiomyocytes with plasmids expressing dominant negative forms of MEK1 (DNMEK1), an upstream activator of p42 MAPK (Lavoie et al., 1996), Akt (DNAkt) (Kotani et al., 1999) and MEKK6 (DNMEKK6), an upstream activator of p38 MAPK (Pandey et al., 1999). The use of dominant negative kinases ensures that only the specific kinases will be made redundant. After overnight transfection the cells were treated for an hour with the peptides, and undergone simulated ischaemia reoxygenation as before.

The presence of DNMEK1 was able to completely abolish the cardioprotective effects of all three peptides (2-way ANOVA, $p<0.05$) (Fig. 5.2B). In a similar manner DNAkt was able to attenuate the cardioprotection of all peptides (Fig. 5.2B) (2-way ANOVA, $p<0.05$). In contrast, the presence of DNMEKK6 had no effect on the cardioprotective effects of the peptides, and in addition, it improved the viability of cardiomyocytes without the presence of peptides (Fig. 6B).

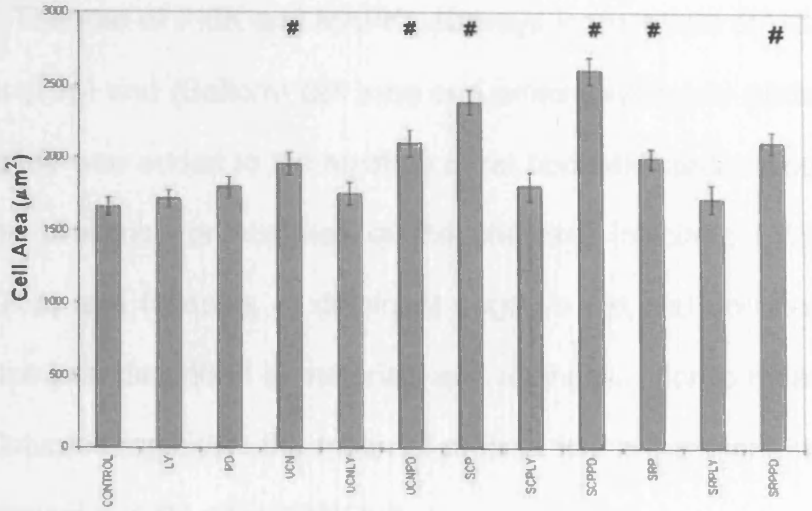
These results, in combination with the chemical inhibitor experiments, clearly show the dependence of all three peptides on activation of both p42/44 MAPK pathway and Akt, in order to elicit their cardioprotective effects.

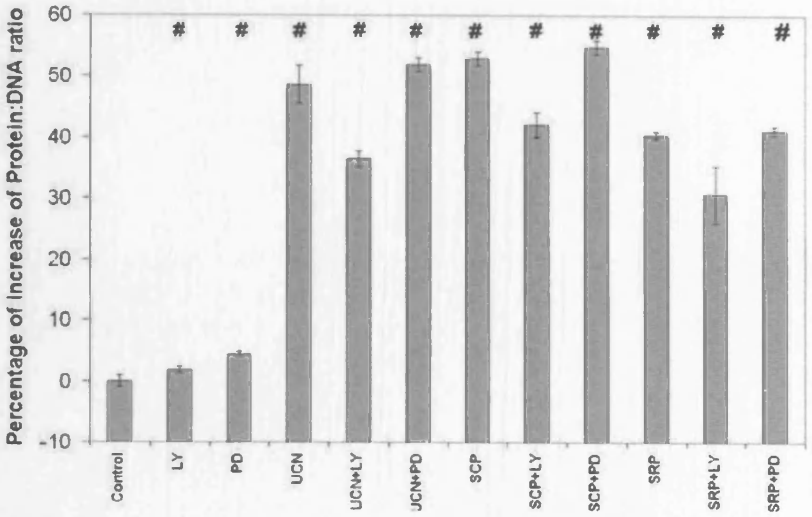
5.2.3 Hypertrophic effects of UCN are attenuated by Akt inhibition, but not by MAPK p42/44 inhibition

UCN peptides have been shown to elicit their cardioprotective effects through activation of MAPK p42/44 and Akt (Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al. 2002b; Dautzenberg and Hauger, 2002; Railson et al., 2002; Schulman et al., 2002). Although, the effects of MAPK activation by UCN have been shown not be involved in hypertrophy (Railson et al., 2000), the role of Akt activation in this context has not been studied. For these reason we elected to study the effect of Akt activation by the UCN peptides on cell size, protein synthesis and natriuretic peptide induction.

When the PI3K pathway was blocked by the chemical inhibitor LY294002, we observed an inhibition on the cell area increase by the UCN peptides (Fig. 5.3 Top). In contrast, chemical inhibition of MAPK caused an increase on the cell area in the presence of the peptides, which was significant in the case of SCP (MANOVA, $p<0.05$ versus SCP) (Fig. 5.3 Top).

Similarly, when we examined the effect of the chemical inhibition of MAPK and PI3K on protein synthesis, we observed that only inhibition of the PI3K pathway was able to attenuate the increase in the protein to DNA ratio by the UCN peptides (Fig. 5.3 MIddle).


Cell Area (μm²)
3000
2500
2000
1500
1000
500
0
#
CONTROL
LY
PD
UCN
UCNLY
UCNPD
SCP
SCPLY
SCPPD
SRP
SRPLY
SRPPD


Percentage of Increase of Protein:DNA ratio
60
50
40
30
20
10
0
-10
#
Control
LY
PD
UCN
UCN+LY
JCN+PD
SCP
SCP+LY
SCP+PD
SRP
SRP+LY
SRP+PD

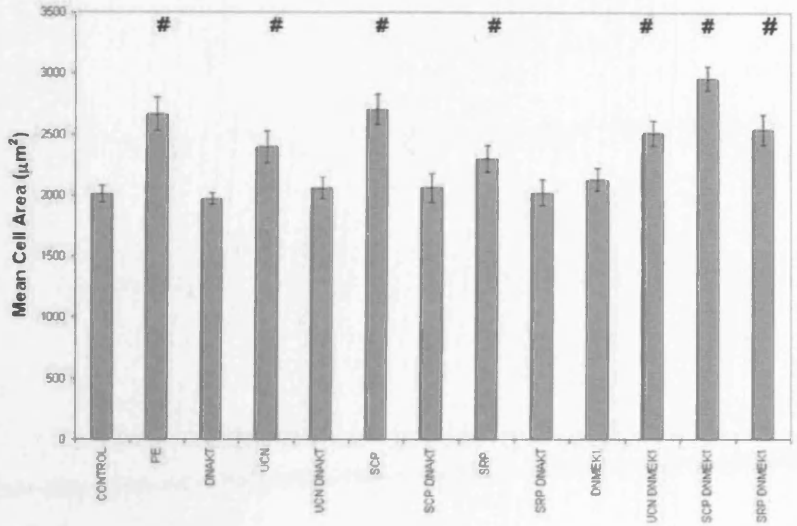

Mean Cell Area (μm²)
3500
3000
2500
2000
1500
1000
500
0
#
CONTROL
PE
DNAKT
UCN
UCN DNAKT
SCP
SCP DNAKT
SRP
SRP DNAKT
DNMEK1
UCN DNMEK1
SCP DNMEK1
SRP DNMEK1

Figure 5.3. The role of PI3K and MAPK pathways in the effect of UCN peptides on cell area (Top) and (Bottom) cell area and protein synthesis (Middle). 10^{-8} M of each peptide was added to the medium of rat neonatal cardiomyocytes for 48 hours in the presence or absence of the chemical inhibitors LY294002 and PD98059 (Top) and (Middle), or dominant negative Akt and dominant negative MEK1 (Bottom) as described in materials and methods. prior to measuring their cell area. Columns represent the mean of at least four experiment and bars are SEM. # represent $p<0.05$ after MANOVA.

Interestingly, unlike the effect of PI3K inhibition on cell area, inhibition of PI3K in the presence of the peptides failed to bring the levels of protein:DNA ratio to control levels observed in the absence of the peptides, indicating that other pathways might be involved in the regulation of protein synthesis by the peptides.

To find out whether Akt, a kinase downstream of PI3K, was implicated in the hypertrophic effects of the peptides, we transfected cells with a dominant negative Akt construct or a dominant negative MEK1 construct (MEK1 is an upstream kinase of p42/44). Once more, only blocking of Akt was able to attenuate the increase in cell area by the UCN homologues (Fig. 5.3 Bottom).

The above results demonstrate that Akt activation is necessary for the increase in cell size and induction of protein synthesis by the UCN peptides. Finally, we examined whether Akt is involved in the induction of ANP and BNP genes by UCN. Blocking PI3K by LY294002, attenuated the induction of ANP and BNP by all three peptides. It is of interest that the levels of the natriuretic peptides were similar to control levels when the peptides were administered in the presence of LY294002 (MANOVA $p<0.05$ for LY294002 plus peptide groups vs control) (Fig. 5.4), indicating that the PI3K pathway is of major importance in the induction of the hypertrophic effects of the UCN peptides.

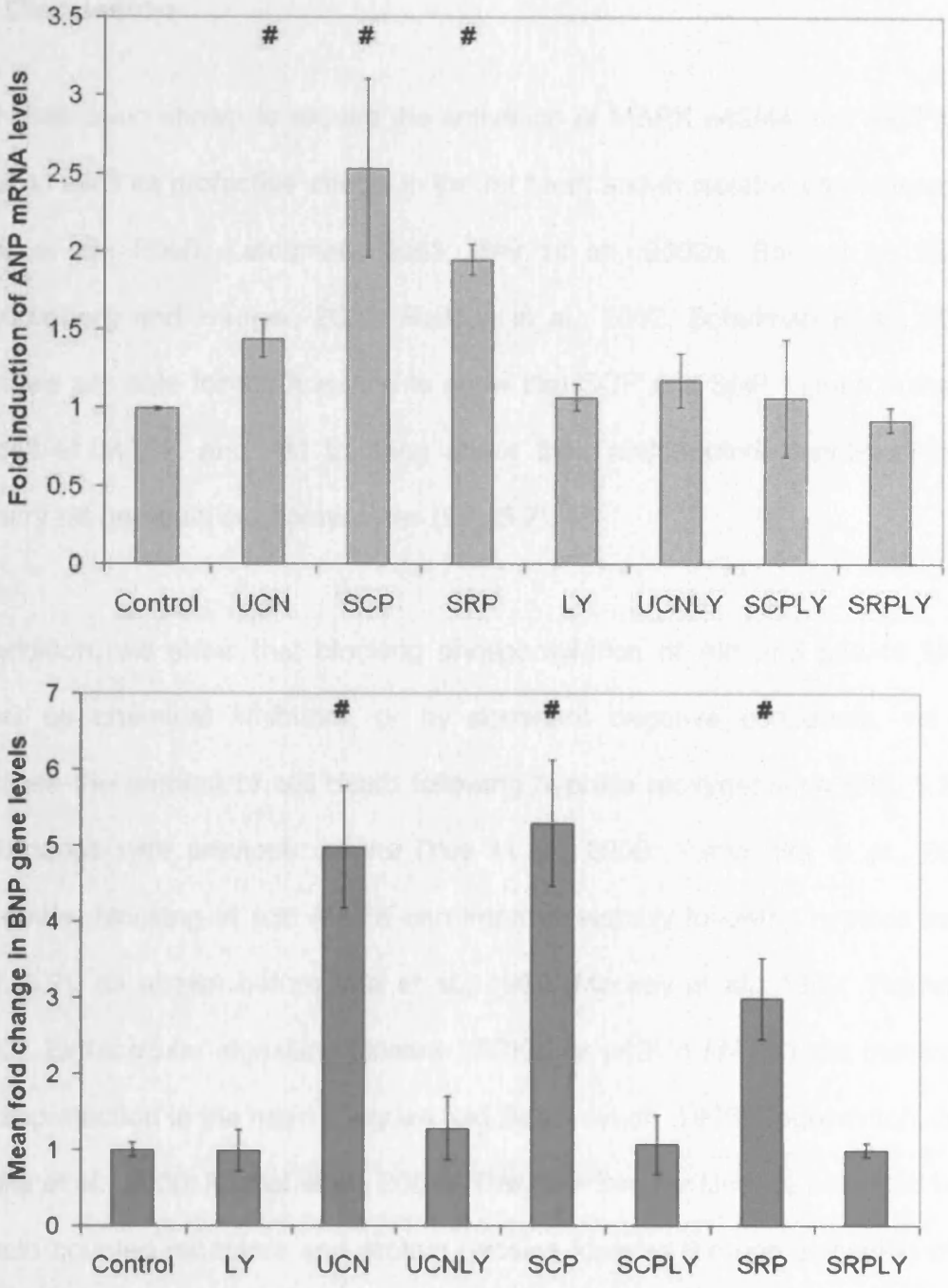


Figure 5.4. The role of PI3K pathway in the induction of (A) ANP gene and (B) BNP gene. 10^{-8} M of each peptide was added to the medium of rat neonatal cardiomyocytes for 48 hours, prior to measuring the changes on gene expression by real time PCR. Columns represent the mean fold change over control of four experiment and bars are SEM. # represent $p<0.05$ after MANOVA.

5.3 Discussion

UCN has been shown to require the activation of MAPK p42/44 and Akt/PKB in order to elicit its protective effects in the rat heart and in isolated cardiomyocytes (Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al. 2002b; Dautzenberg and Hauger, 2002; Railson et al., 2002; Schulman et al., 2002). Here we are able for the first time to show that SCP and SRP require activation of p42/44 MAPK and Akt to bring about their antiapoptotic functions in the primary rat neonatal cardiomyocytes (Fig. 5.2).

In addition, we show that blocking phosphorylation of Akt and p42/44 MAPK either by chemical inhibitors, or by dominant negative constructs, we can increase the amount of cell death following hypoxia reoxygenation (Fig. 5.2), in accordance with previous results (Yue et al., 2000; Yamashita et al., 2001). Moreover, blocking of p38 MAPK can improve viability following hypoxic insults (Fig. 5.2), as shown before (Ma et al., 1999; Mackay et al., 1999; Yue et al., 2000). Extracellular signalling kinases (ERKs, or p42/44 MAPK) are involved in cardioprotection in the heart (Sugden and Bogoyevitch, 1995; Bogoyevitch, 2000; Yaoita et al., 2000; Michel et al., 2001). They are Ser/Thr kinases activated by G-protein coupled receptors and protein tyrosine kinases through activation of ras and raf (Yaoita et al., 2000; Johnson and Lapadat, 2002). Downstream effectors of their survival effects include Bcl-xL (Mori et al., 2003) and they have been shown to be required for prevention of cytochrome C release from the mitochondria (Erhardt et al., 1999).

Furthermore, we are able to demonstrate that SCP is a more potent activator of the p42/44 MAPK, followed by UCN and SRP last (Fig. 5.1). This pronounced activation of p42/44 MAPK by SCP can also explain the potent cardioprotective effects of SCP, and can further strengthen the notion that SCP is binding to CRFR2 with higher affinity than UCN. It has been previously shown in myometrial cells that phosphorylation of the MAPK p42/44 is a downstream event that follows the activation of CRFR1α and CRFR2β (Grammatopoulos et al., 2000).

Our results demonstrate that UCN homologues require activation of both p42/44 MAPK and Akt, in order to protect cardiomyocytes from hypoxia/reoxygenation injury (Fig. 5.1 and 5.2). This would suggest that the two pathways might be linked. It has been shown before that cAMP can stimulate Akt in 293-EBNA cells and in WIF-B9 cells through activation of G-protein coupled receptor protein G$\beta\gamma$ (Sable et al., 1997; Kagawa et al., 2002).This is possibly through a direct interaction of PI3K with Ras as shown before (Rubio et al., 1997). Taken together it would mean that G-protein coupled receptor activation by the UCN homologues can induce both pathways at the same time through activation of Ras. This model could explain the increase in p42/44 phosphorylation observed after treatment with SCP and UCN in the presence of LY294002 (Fig. 5.1). However this had no effect in protection (Fig. 5.2), suggesting that UCN peptides need activation of downstream protective effectors regulated by both pathways.

Finally, inhibition of p38 MAPK increased phosphorylation of p42/44 MAPK (Fig. 5.1), in the presence of UCN and SCP. Again, this would suggest a crosstalk between the different pathways. This crosstalk between p38 MAPK and p42/44 MAPK has been shown to occur in epithelial corneal cells, where chemical inhibition of p38 MAPK increased phosphorylation of p42/44 MAPK in the presence of hepatocyte growth factor (Sharma et al., 2003).

Although, previous studies on UCN hypertrophic effects speculated that PKA activation was important for these effects, this is the first report to show a distinct mechanism for the UCN induced hypertrophy. It is clear that PI3K and Akt activation in particular is at least one of the factors required not only for the hypertrophic effects of the UCN peptides, but for cardioprotection as well. Akt activation is known to be an important factor in hypertrophy (Frey and Olson, 2003). Possible downstream effectors include such regulators of hypertrophy as GSK3β and mTOR. CRF has already been shown to regulate GSK3β expression in neuronal cells (Bayatti et al., 2003).

We previously speculated that Akt and MAPK pathways converge at a point downstream of the CRFR2. This notion is strengthened by the results presented here. Akt phosphorylation (Fig. 5.1) was increased after blocking MAPK and in addition, in some of the experiments, blocking MAPK increased protein synthesis and cell area after peptide treatment (Fig. 5.3). These increases were not statistically significant however, apart from the effect of SCP on cell area (Fig. 5.3

Top and Bottom) and in the case of SRP when cells were transfected with DNMEK1 (Fig. 5.3 Bottom). These results indicate that there is a convergence of the two pathways upstream of MEK1 and that the MAPK pathway might act as a negative feedback loop on the Akt pathway stimulation on cell area after the peptide activation of the CRFR2, and probably only after an increased stimulation of Akt, as is the case with SCP and SRP. However, this hypothesis needs to be tested. It has been previously shown that certain isoforms of the PI3K can be activated by Gsq trimeric proteins and also PI3K is able to induce MAPK activation either by interacting with ras, or by activating protein kinase C, which in consequence acts on raf-1 to activate the MAPK pathway (Bondeva et al., 1998; Graness et al., 1998; Murga et al., 1998;Clerk and Sugden 1999; Bayatti et al., 2003; Bayer et al, 2003; Foncea et al., 2000; Wetzker and Bohmer, 2003). In addition, protein kinase A, which is activated by Gs, can stimulate Akt independently of PI3K (Sable et al., 1997; Filippa et al., 1999).

In conclusion it appears that although all three peptides require activation of p42/44 MAPK and of Akt to bring about their cardioprotective effects, only activation of Akt is required for hypertrophy to be induced. This would mean that it might be possible to identify the factor or factors downstream of Akt responsible for the hypertrophic effects and maybe be able to block them in order to pave the way for the use of the peptides as therapeutic agents.

CHAPTER VI

Other mechanisns of cardioprotection by UCN homologues

6.1 Introduction and aims

UCN and its homologues are dependent on activation of p42/44 MAPK and PI3-K/Akt in order to confer their effects, as shown in the previous chapters. However, it was previously shown that UCN cardioprotection (Brar et al., 2002a) requires *de novo* protein synthesis, as the cardioprotective effects of UCN were abolished in the presence of the protein synthesis inhibitor cyclohexamide. This observation led us to suggest that activation of the MAPK and Akt is not the sole required event for UCN cardioprotection. The identity and function of these end-effector proteins is important in not only understanding the mechanism of action of the UCN homologues, but also in the development of novel protective pharmacological compounds that could be used prior or consequent to an ischaemic episode.

The proteins that are examined here were identified and shown to be regulated by UCN through an affymetrix gene chip assay (Lawrence et al., 2002). The three molecules that we selected to examine further are $iPLA_2$, PKCε and Kir 6.1. All three molecules were shown before to be involved in ischaemia reperfusion injury in the heart, but were not associated with UCN. PKC_ε and $iPLA_2$ are proteins that are involved in signalling, whereas Kir6.1 is a subunit of the K_{ATP}

channel. All three molecules are either up- or down- regulated by UCN and we were interested to find out whether SCP and SRP would be affecting those molecules in a similar manner.

6.2 Results

6.2.1 UCN homologues induce PLA_2 expression

$iPLA_2$ is a an enzyme that belongs to the superfamily of esterases that hydrolyse the sn-2 ester bond in phospholipids, releasing arachidonic acid and lyso metabolites, principally lysophosphatidylcholine. Only $iPLA_2$ from all the PLA_2 lipases has been shown to increase its activity during ischaemia/reperfusion, with a resulting increase in its metabolites (Shizume et al., 1997; Daleau et al., 1999; Cummings et al., 2000). The transcript for $iPLA_2$ was shown to be decreased by 2.5 fold by UCN treatment (table 6.1).

We wanted to establish whether the levels of $iPLA_2$ are decreased by UCN and its homologues in rat neonatal cardiomyocytes following a hypoxic insult. For that cells were treated with 10^{-8} M of UCN, SCP or SRP for 24 hours prior to a 4 hour hypoxia and 16 hour reoxygenation. The protein levels were analysed by western blotting with an antibody specific for $iPLA_2$ (Fig. 6.1).

All three peptides attenuate the levels of $iPLA_2$ not only in control condition, but after hypoxia/reoxygenation as well (Fig. 6.1). The levels of the phospholipase were increased after hypoxia/reoxygenation 3 fold approximately. UCN treatment was able to reduce the levels of the enzyme at least 2 fold in control conditions and at least 3-fold reduction was observed after hypoxia/reoxygenation (Fig. 6.1). SRP showed similar levels of $iPLA_2$ reduction to UCN both under control and hypoxia/reoxygenation conditions. SCP showed the greatest effect on the levels of $iPLA_2$ (Fig. 6.1).

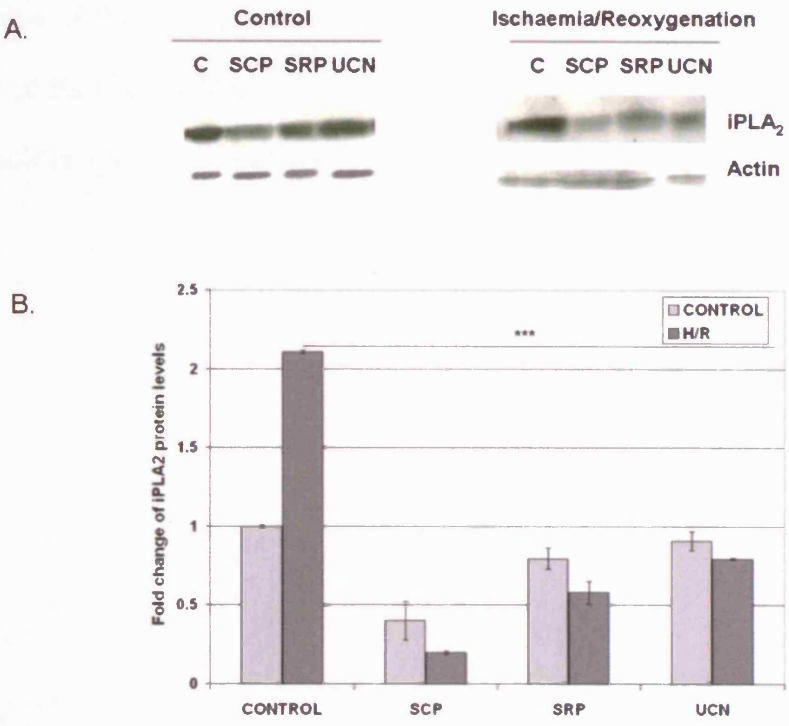


Figure 6.1. UCN homologues attenuate $iPLA_2$ protein levels in rat neonatal cardiac myocytes. Cells were treated for 24 hours with 10^{-8} M of UCN, SCP or SRP prior to 4 hours hypoxia and 16 hour reoxygenation. **A.** Western blot of $iPLA_2$ from protein isolated from rat neonatel cardiomyocytes treated with UCN homologues with and without hypoxia/reoxygenation insult. **B.** Densitometric analysis of at least 3 western blots. Actin was used for normalisation. Values are the average of at least three experiments with their standard deviation represented by the bars. Stars represent statistical significance (2-way ANOVA, $p<0.05$, followed by Bonferroni post-hoc analysis).

The above results establish that $iPLA_2$ is a target of the UCN homologous peptides and that the administration of the peptides can lead to a pronounced reduction of the levels of the $iPLA_2$ enzyme, causing a possible reduction in the levels of its metabolites AA and LPC, minimising the deleterious effects of the metabolites on the cardiac cell.

6.2.1.1 UCN homologues induced protection is attenuated by LPC and increased by BEL

Lysophosphatidylcholine (LPC) one of the metabolites of $iPLA_2$ has been shown to be involved in ischaemia/reperfusion injury. For this reason we treated cells with $5x10^{-9}$ M of LPC for 24 hours in the presence or absence of UCN homologous peptides and analysed the effect of the metabolite on apoptotic cell death by TUNEL assays.

LPC showed devastating effects on rat neonatal cardiomyocytes causing almost 70% of the cells to die by apoptosis (Fig. 6.2). The effects of LPC were attenuated when the cells were treated at the same time with 10^{-8} M of any of the three peptides UCN, SCP or SRP. However the amount of death present was still greater than in the control cells, indicating that the effect of the peptides is on the $iPLA_2$ enzyme, rather than the metabolites and their downstream targets. The amount of cell death among the three different peptides was comparable (MANOVA, $p>0.05$) and none of the three peptides appears to work significantly better against LPC induced damage.

To determine the role of the increase of endogenous levels of LPC after a hypoxic insult, cells were treated with bromoenol lactone (BEL) an inhibitor of $iPLA_2$, in the presence or absence of UCN homologues for 24 hours prior to hypoxia/reperfusion. Subsequently, the cells were fixed and analysed by TUNEL to determine the amount of apoptotic cell death occurring (Fig. 6.3).

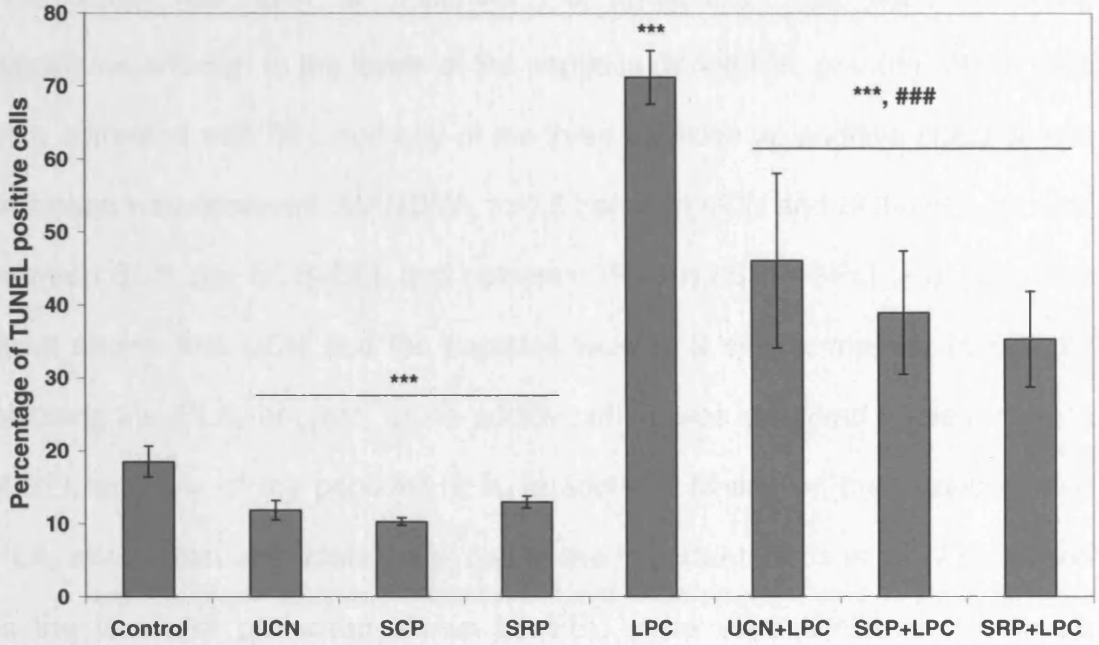


Figure 6.2. Effect of LPC on UCN peptide cardioprotective effects. Rat neonatal cardiomycytes were treated with $5x10^{-9}$ M LPC and 10^{-8} M of UCN, SCP or SRP and the effects on cell survival were determined by TUNEL assay. Columns represent the average of at least six experiments, and bars represent standard deviation of the groups. ***: $p<0.05$ compared to control group, ###: $p<0.05$ compared to LPC group (MANOVA).

BEL alone was able to minimise the amount of cell death following hypoxia/reperfusion to the levels of the peptides (MANOVA, $p>0.05$). When cells were cotreated with BEL and any of the three peptides no additive effect on cell protection was observed (MANOVA, $p>0.5$ between UCN and UCN+BEL groups, between SCP and SCP+BEL and between SRP and SRP+BEL) (Fig. 6.3). This result shows that UCN and the peptides work in a similar manner to BEL by inhibiting the $iPLA_2$ enzyme, as no additive effect was observed in the presence of BEL and any of the peptides. It is, in addition, hinting on the possibility that $iPLA_2$ attenuation and inhibition is one of the important steps in UCN protection as the levels of protection shown by BEL alone were similar to the levels conferred by any of the three peptides alone.

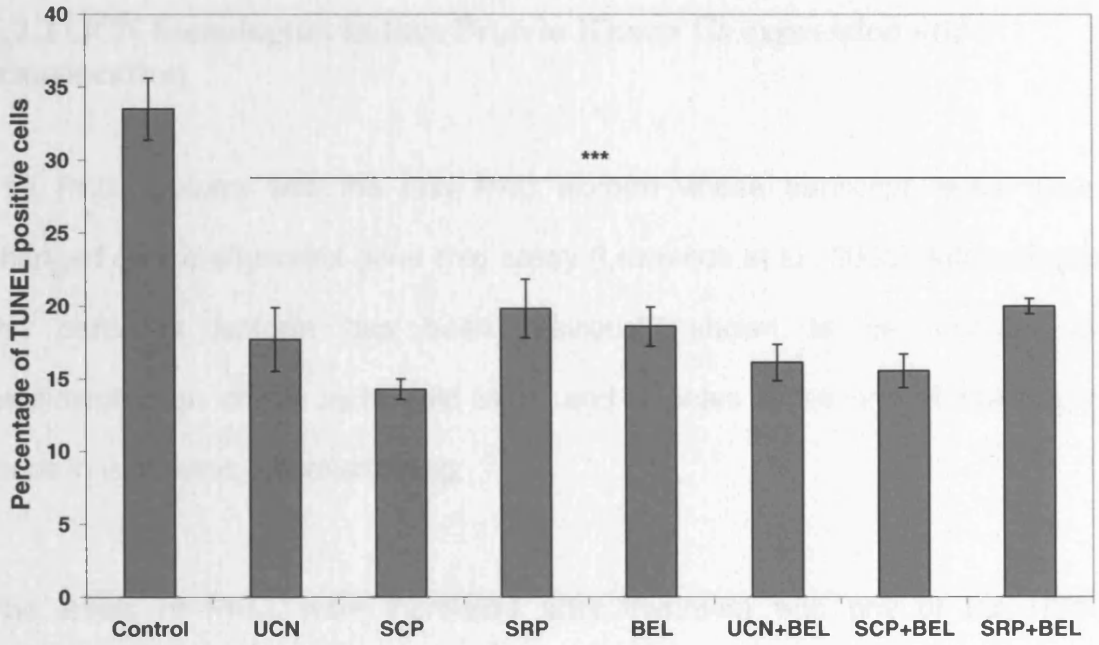


Figure 6.3. Effect of BEL on the cardioprotective effects of the UCN homologues. Rat neonatal cardiomyocytes were treated with 10^{-6} M of BEL and 10^{-8} M of UCN, SCP or SRP prior to a four hypoxia-sixteen hour reoxygenation period, and then cell death was analysed by TUNEL assay. Columns represent the average of at least six experiments with bars representing the standard deviation in the group. ***: MANOVA, $p<0.05$ vs Control.

6.2.2 UCN homologues induce Protein Kinase Cε expression and translocation

The PKC_{ε} isoform was the only PKC isoform whose transcript levels were changed on the affymetrix gene chip assay (Lawrence et al., 2005). Interestingly the particular isoform has been previously shown to be involved in cardioprotection during ischaemic injury and appears to be one of the major steps in ischaemic preconditioning.

The levels of PKC_{ε} were increased after treatment with any of the UCN homologous peptides (Fig. 6.4). The peptides had no effect on the levels of another PKC isoform, PKC_{δ} (Fig. 6.4). Once more SCP had more pronounced effects on the levels of PKC_{ε} than any of the other two peptides, with UCN being the next most potent peptide (Fig 6.4).

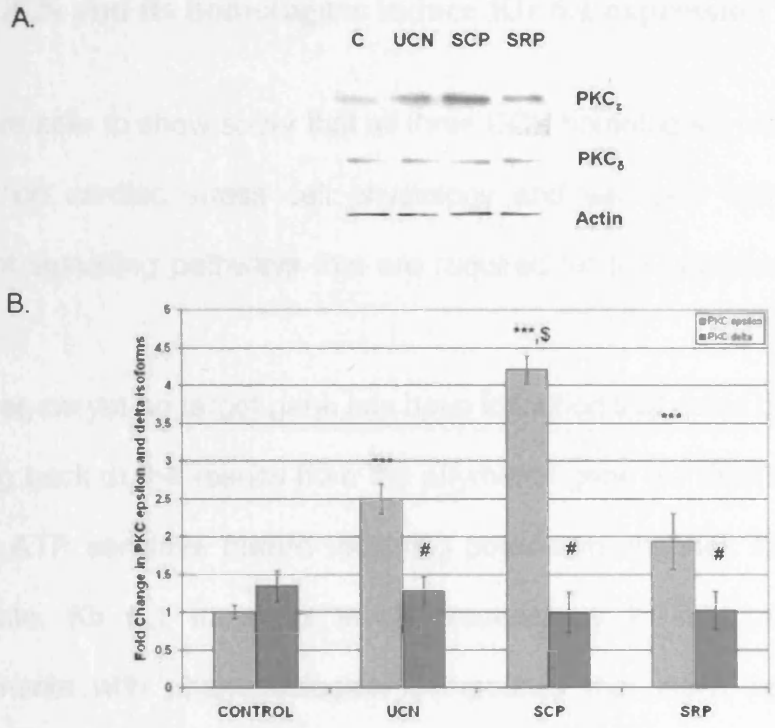


Figure 6.4. UCN homologues peptides induce PKC_ε expression. A. Western blot analysis for PKC_ε and PKC_δ of protein extracts from rat neonatal cardiomyocytes treated for 24 hours with 10^{-8} M of UCN, SCP and SRP. Actin was used for normalisation. B. Densitometric analysis of at least three blots of the western blot. Columns represent the means and the bars the SD. Stars: MANOVA, $p<0.01$ vs PKC_ε control, #: MANOVA $p>0.05$ vs PKC_δ control, and $: MANOVA, $p<0.01$ vs all other homologues (Bonferroni post-hoc analysis).

6.2.3 UCN and its homologues induce Kir 6.1 expression

We were able to show so far that all three UCN homologues have a considerable impact on cardiac stress cell physiology and we were able to identify four different signalling pathways that are required for the peptides to produce their effects.

However, as yet no target gene has been identified that could be an end effector. Looking back at the results from the affymetrix gene we decided to concentrate on the ATP sensitive inward rectifying potassium channel, Kir 6.1, as a good candidate. Kir 6.1 transcript levels changed by 2.6-fold, and a number of experiments with pharmacological compounds that either open or close the channel, have shown that the opening of the channel has cardioprotective effects against ischaemia and is likely to be involved in ischaemic preconditioning (Bernardo et al., 1999; Takashi et al., 1999; O'Rourke, 2000; Takano et al., 2000). Moreover, the effect on the Kir 6.1 subunit seems to be a specific one, as the other subunits, Kir6.2 and SUR2, showed no change in the affymetrix gene chip assay (Lawrence et al., 2002). This specificity of action of UCN on the channel was confirmed by reverse transcription PCR, RNA slot blots and westerns (Lawrence et al., 2002), where no change in Kir 6.2, SUR1 or SUR2 was shown after 24 hour of UCN treatment.

To compare the effects of the UCN homologues we treated rat neonatal cardiomyocytes for 24 hours with UCN, SCP or SRP and analysed their effects

on the Kir 6.1 protein levels by western blotting (Fig. 6.5). All three peptides were able to induce Kir 6.1 expression after a 24 hour treatment with SCP inducing a 4.5 fold increase compared to a 3 fold increase by UCN and a 1.5 fold by SRP. Once again SCP proves to be the most potent peptide of the three followed by UCN. In addition none of the peptides had any effect on the levels of the Kir 6.2 isoform (Fig. 6.5).

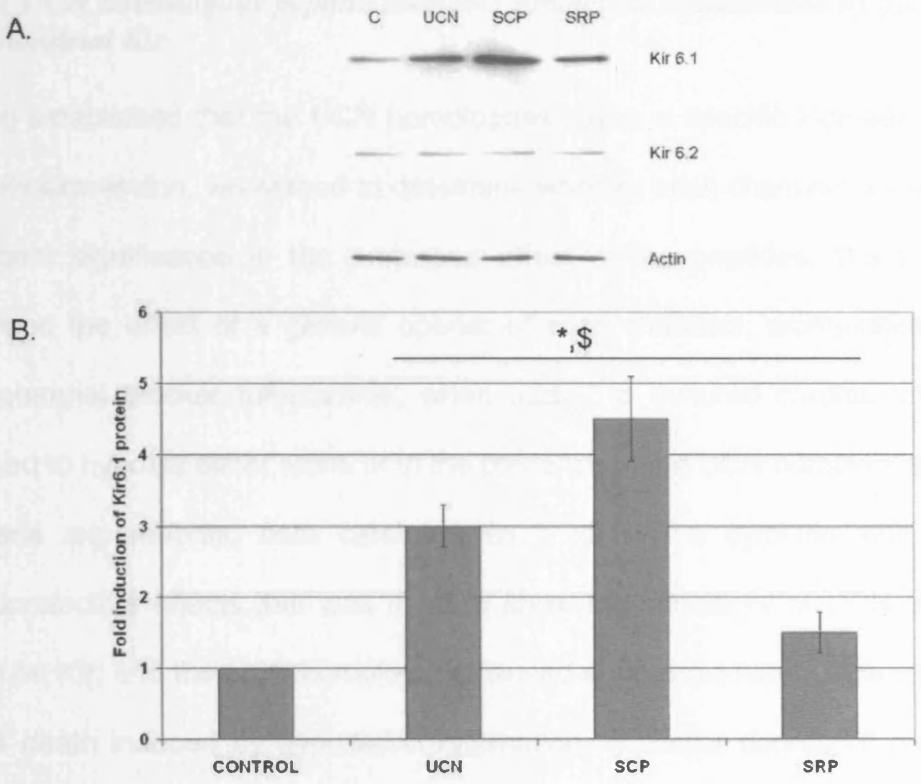


Figure 6.5. Effect of the UCN homologues on the protein levels of Kir 6.1. A.Rat neonatal cardiomyocytes were treated with 10^{-8} M of Ucn, SCP or SRP for 24 hours and the protein levels of Kir 6.1 and 6.2 were analysed by western blotting. Actin was used for normalisation. B. Densitometric analysis of at least three Kir6.1 blots. Columns represent the means and the bars the SD. Stars: MANOVA, $p<0.05$ vs control and $: MANOVA, $p<0.05$ vs all other homologues (Bonferroni post-hoc analysis). No densitometry for Kir 6.2 is presented as there were no statistical significant differences between the treated groups and the control sample and no differences among the treated groups.

6.2.3.1 UCN homologous peptide-mediated protection is attenuated by blocking mitochondrial Kir

Having established that the UCN homologues cause a specific increase in K_{ATP} channel expression, we wished to determine whether such channels were of any functional significance in the protective effect of the peptides. We therefore examined the effect of a general opener of such channels, cromakalim or the K_{ATP} channel blocker tolbutamide, when added to cultured cardiac myocytes exposed to hypoxia either alone or in the presence of the UCN homologues.

In these experiments, both cardiotrophin 1 (CT-1), a cytokine with known cardioprotective effects that was used to show the specificity of UCN peptides action on Kir, and the UCN homologues caused an approximately 50% reduction in cell death induced by hypoxia/reoxygenation. A similar degree of protection was observed with the K_{ATP} channel opener cromakalim, whereas addition of tolbutamide enhanced the levels of cell death (Fig. 6.6). Most interestingly, addition of tolbutamide attenuated the protective effects of the UCN homologues, whereas it had no effect on the protective effects of CT-1, underlying the fact that the effects of the UCN homologues on the K_{ATP} channel are specific.

Tolbutamide is generally considered to be a sarcolemmal channel blocker, although inhibition of mitochondrial K_{ATP} channels has been reported. However, it is generally believed that it is the mitochondrial channel that is respnsible for the cardioprotective effect of K_{ATP} channel opening. We therefore wished to determine wheher the effect of the UCN homologues would be prevented by a selective mitochondrial K_{ATP} channel blocker, 5-hydroxy decanoate (5-HD). In

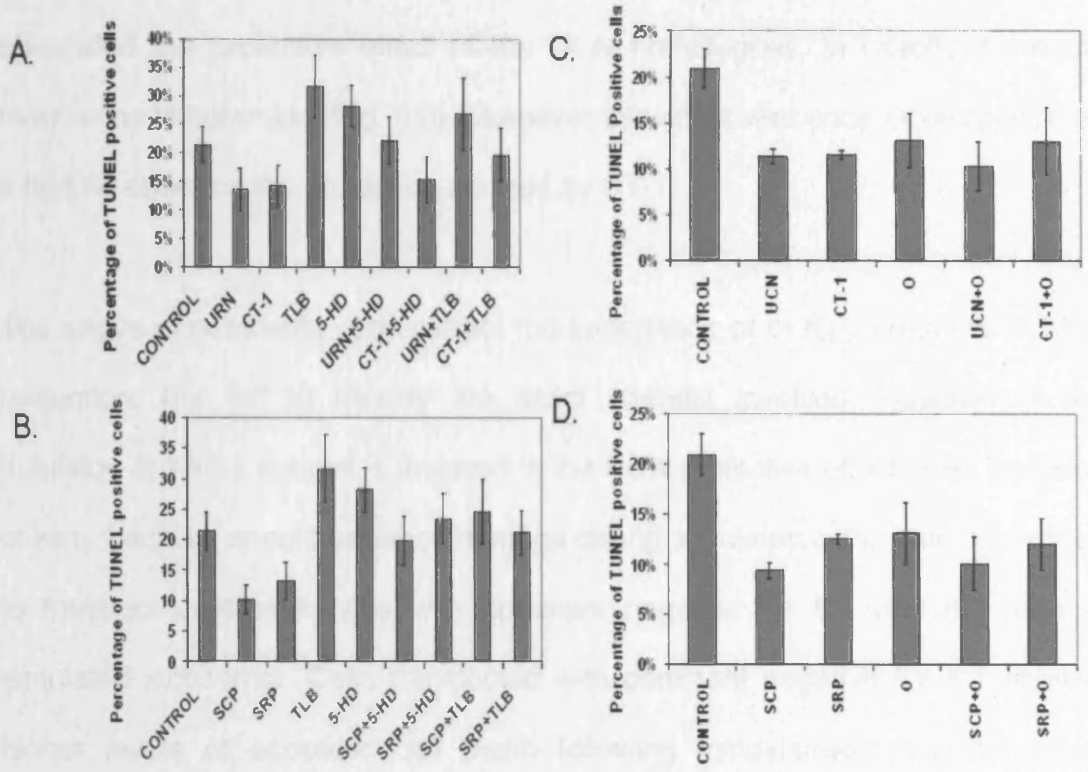


Figure 6.6. Effect of K_{ATP} channel blockers (A, B) and opener (C, D) on the cardioprotective effects of the UCN homologues. Rat neonatal cardiomyocytes were exposed to simulated ischaemia/reoxygenation injury after treatment with 10^{-8} M of a UCN homologue, 10^{-6} M CT-1, 10^{-8} M cromakalim (O), 10^{-7} M tolbutamide (TLB), or 5-hydroxydecanoate (5-HD) for 2 hours. Values are averages of at least 3 experiments; bars, SD.

these experiments, 5-HD induced enhanced cell death in cardiac myocytes exposed to simulated ischaemia. Most interestingly, 5-HD treatment completely abrogated the protective effect of the UCN homologues, in exactly the same manner as tolbutamide (Fig. 6.6). Moreover this effect was once more specific as it had no effect on the protection afforted by CT-1.

The above experiments demonstrate the importance of of K_{ATP} channels in UCN protection, but fail to identify the exact channel involved. However, if the induction of Kir6.1 subunit is involved in the UCN protective effect, then inhibition of Kir6.1 activity should enhance damage during simulated ischaemia. We chose to transfect cardiomyocytes with dominant negative Kir 6.1 and 6.2 prior to simulated ischaemia. Cells transfected with dominant negative Kir 6.1 showed higher levels of apoptotic cell death following hypoxia/reoxygenation injury, significantly higher levels of cell death compared to cells transfected with Kir 6.2 (Fig. 6.7). Furthermore, addition of UCN homologous peptides failed to rescue cells transfected with dominant negative Kir 6.1, strengthening the involvement of Kir in the protective mechanisms of UCN (Fig. 6.7).

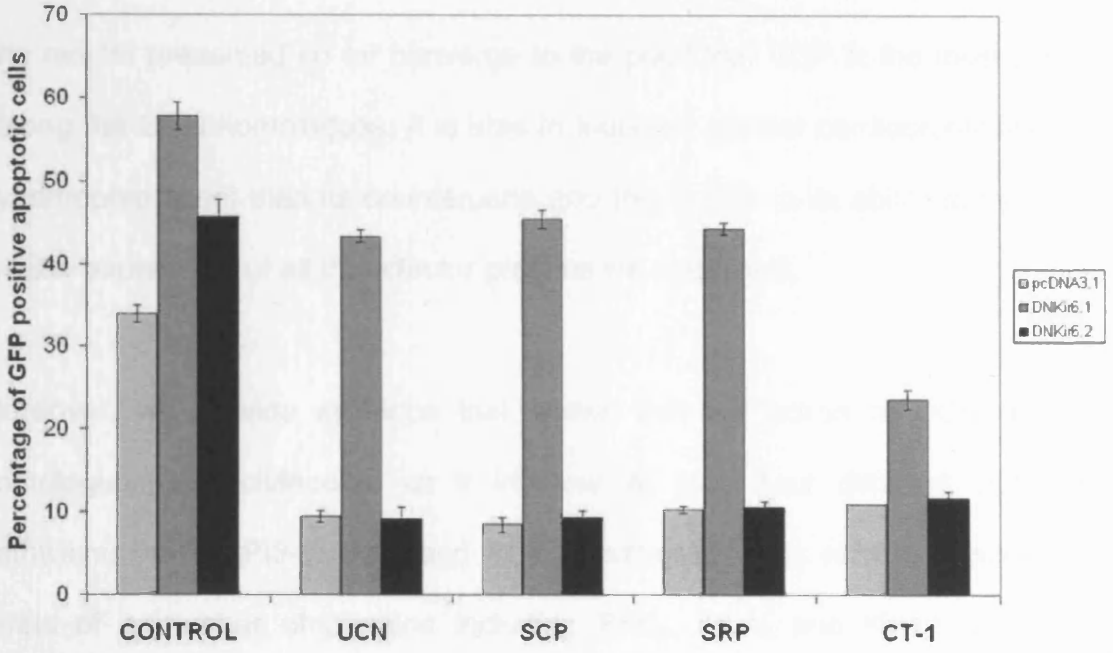


Figure 6.7. Effect of Kir 6.1 on cardiomycyte survival after hypoxia/reoxygenation and on UCN cardioprotection. Rat neonatal cardiomyocytes were transfected with 0.5μg of GFP and 0.5 μg of either dominant negative Kir 6.1 or dominant negative Kir 6.2 and treated with 10^{-8} M a UCN homologue or 10^{-6} M of CT-1 and analysed by TUNEL. Values are the averages of at least three experiments; bars SD.

6.3 Discussion

The results presented so far converge to the point that SCP is the most potent among the UCN homologues. It is able to induce a greater cardioprotective and hypertrophic effect than its counterparts and this is due to its ability to induce a greater expression of all the effector proteins we examined.

Moreover, we provide evidence that shows that the action of UCN and its homologues is multifaceted as it involves at least four different signalling pathways: MAPK, PI3-K, PKC and PLA_2. Additionally, it is able to regulate the levels of a number of proteins including PKC_ε, $iPLA_2$ and Kir6.1, and thus affecting cell signalling, lipid metabolism and ion homeostasis, in order to assist the cardiomyocyte in conditions of stress.

For the first time we are able to show target proteins of UCN and to exhibit evidence that LPC and Kir 6.1 are involved in cardiomyocyte survival after a hypoxic stress.

UCN and its homologues were able to downregulate the levels of $iPLA_2$, as expected from the affymetrix gene results (Lawrence et al., 2002). However, the effect of SCP on the levels of $iPLA_2$ was even more pronounced. $iPLA_2$ is a phospholipase that cleaves the sn-2 ester bond of phospholipids and it has been involved in apoptosis (Jaattela et al., 1995; Atsumi et al., 1998; Zhao et al.,

2001a; Zhao et al., 2001b). Additionally, in RAW264.7 cells and in mouse peritoneal macrophage cultures, $iPLA_2$ was shown to be the form of phospholipase involved in oxidative stress (Martinez and Moreno, 2001). Only $iPLA_2$ was shown to be significantly active after H_2O_2 stress, and that the arachidonic acid release, due to increased activity of $iPLA_2$, was dose dependent to H_2O_2. Arachidonic acid release by H_2O_2 was blocked by BEL. In addition, antisense $cPLA_2$ had no effect on the arachidonic acid release (Martinez and Moreno, 2001).

UCN and its homologues might be able to reduce apoptotic cell death by attenuating expression of $iPLA_2$ and minimising the increase in the concentration of the metabolites AA and PLC after ischaemia/reperfusion. We observed that BEL alone is able to reduce the amount of apoptotic cell death after hypoxia/reoxygenation, but addition of UCN or any of the homologues showed no additive effects, suggesting that BEL and UCN homologues act in a similar mechanism; Similarly UCN and its homologues attenuated the amount of cell death caused by the product of $iPLA_2$, LPC, even if they failed to reduce the amount of cell death to control levels.

We also examined the effects of UCN and its homologues on the levels of another signaling molecule, PKCε. PKCε is an isoform that has been shown to be involved in ischaemia and in cardiac hypertrophy. It appears that the end result of PKC activation in the heart is dependent on the levels of PKCε and

PKCδ. More PKCε leads to protection whereas more PKCδ leads to death. In our experiment we showed that all three homologues induce expression of PKCε without affecting the levels of PKCδ, introducing another mechanism of protection by the UCN peptides. It has been recently shown that PKC_ε inhibition with specific RACK peptide inhibitors, attenuated the cardioprotection conferred by UCN, exhibiting the importance of PKC_ε translocation for the protective effects elicited by UCN (Lawrence et al., 2005). Interestingly, it was shown that PKC epsilon is localised in the mitochondrial membrane, suggesting a possible role in regulated K_{ATP} channels and $iPLA_2$ on the mitochondrial compartment and being the main orchestrator of the UCN effects on maintaining mitochondrial integrity and abolishing apoptosis caused by the mitochondrial pathway.

Finally the third protein we selected to study was Kir6.1, a subunit of the ATP sensitive inward rectifying potassium channel, K_{ATP}. It was previously shown that K_{ATP} opening is cardioprotective and that K_{ATP} is involved in ischaemic preconditioning. Our results show that the peptides are able to induce Kir 6.1 expression, which is specific, as it had no effect on the Kir isoform 6.2 or on the sulfonylurea receptor SUR. Additionally, Kir6.1 expression is a requirement for cardioprotection by the peptides, as dominant negative Kir6.1 induced death even in the presence of the peptides. There has been considerable discussion, whether the sarcolemmal or the mitochondrial subtype of Kir6.1 is involved in cardioprotection. We show that the effects of a general K_{ATP} (TLB) and a mitochondrial K_{ATP} (5-HD) blocker had similar effects on cell death, suggesting

the dominant role of the mitochondrial K_{ATP} channel on protection, and the possibiltity that Kir6.1 is the specific type involved in the formation of the mitochondrial K_{ATP} channel.

CHAPTER VII

SAG is induced by UCN homologues

7.1 Introduction and aims

Apoptosis is characterised by activation of caspases that orchestrate a cascade of events that lead to cellular degradation. A main manifestation of apoptosis is nuclear condensation and DNA degradation, a process that is used in TUNEL staining to visualise apoptotic cells.

There is a great need to characterise the pathways that regulate apoptosis in the heart, in order to establish new therapeutic strategies in failing hearts. There is considerable knowledge of the pathways that lead to activation of caspases from extracellular signals and from mitochondrial damage. There is also considerable interest in genes that are characterised as anti-apoptotic such as the inhibitors of apoptosis (IAPs) which inhibit the onset of apoptotic cascades by interacting with caspases and preventing their activation (Goyal, 2001).

One such gene that was recently identified was named Sensitive to Apoptosis Gene (SAG) and was cloned as a factor that is induced after oxidative stress (Duan et al., 1999). SAG is a 13 kD protein, containing a $C_2H_2C_4$ Zn-RING finger motif. It was recently shown to be expressed in hypoxic conditions in the brain and to co-localise with newly produced radical oxygen species (ROS), indicating the function of SAG as a possible antioxidant. The RING finger motif of SAG has

been shown to be important for its antiapoptotic function, as mutants of the SAG at the RING motif are unable to protect from apoptosis when overexpressed (Yang et al., 2001).

SAG is shown to be localised in the cytoplasm and nucleus of the cell and is ubiquitously expressed in many different organs and tissues, but predominantly in heart, brain and the gonads (Duan et al., 1999).

Additionally, SAG is overexpressed in several carcinomas (Sun, 1999; Huan et al., 2001; Sasaki et al., 2001), although overexpression of SAG alone is not enough to induce neoplastic transformation of cells (Huang et al., 2001). SAG was shown to promote S-phase and serum free growth of cells (Duan et al., 2001) and downregulation of SAG, by transient transfection of an antisense SAG construct in cancer cells resulted in reduction of the proliferation rate of these cells (Huang et al., 2001) demonstrating the importance of SAG in control of cell proliferation. Furthermore, the involvement of SAG in cell proliferation was demonstrated in yeast, where it was shown by gene chip profiling to regulate expression of components of the cell cycle (Sun, 1999; Swaroop et al., 2000).

SAG belongs to a family of proteins known as regulator of Cullins (ROC) that bind through cullin to the SCF (Skp, Cullin, F-box) multisubunit proteins (Ohta et al., 1999). One proposed mechanism by which SAG might elicit its antiapoptotic effects is by regulating $p27^{Kip1}$ (Ohta et al., 2000; Duan et al., 2001).

Although SAG is highly expressed in the heart (Duan et al. 1999) its role in cardiac cells has not been previously characterised. In view of the critical clinical importance of ischaemia in the heart, we tested whether SAG is able to protect cardiomyocytes from ischaemic insults.

In addition, we were interested to show whether UCN and its homologues had any effect on SAG, as it is a molecule that is expressed in the heart and brain and it has antiapoptotic properties.

7.2 Results

7.2.1 SAG induction by UCN peptides

SAG is a novel gene with antiapoptotic properties and we wanted to establish whether the gene is induced by UCN and its homologues. Primary rat neonatal cardiomyocytes were treated with 10^{-8} M of each peptide prior to determining the levels of SAG protein in the cells by western blotting. UCN and its peptides failed to induce SAG after 24 hours, but the levels of SAG protein doubled after 36 hours of peptide addition (Fig. 7.1).

Although, there was SAG induction by the peptides in neonatal cells this induction was only detectable at the 36 hour mark, showing that SAG is a late target of UCN peptide action in the neonatal cardiomycyte. The role of SAG induction by UCN needs to be further investigated.

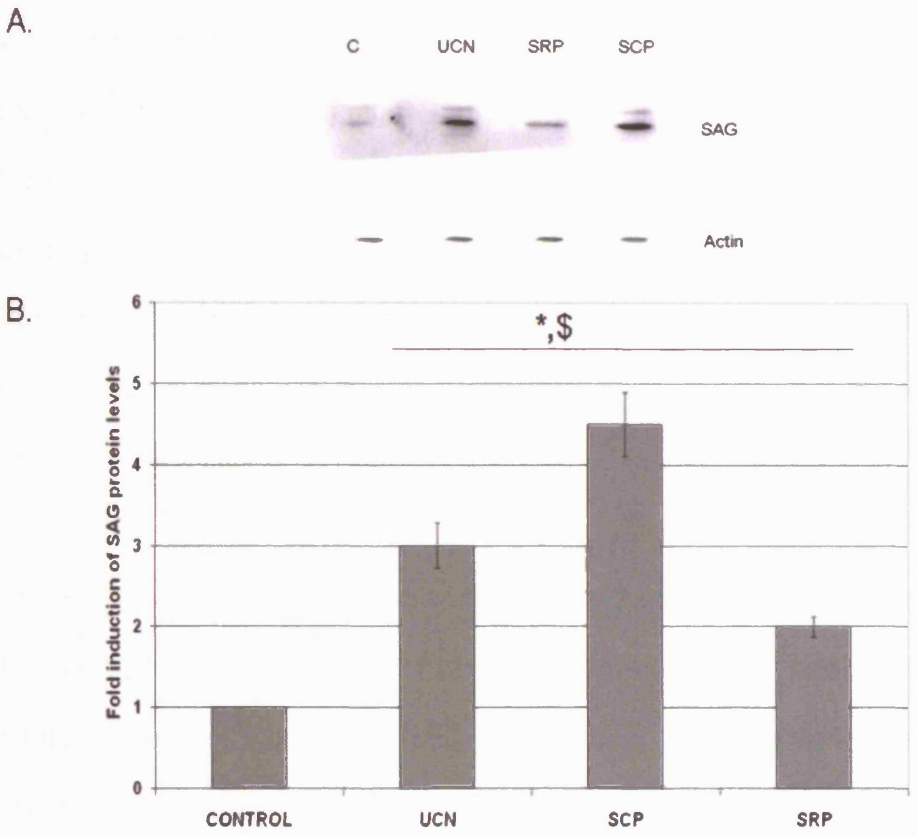


Figure 7.1. Western blot analysis showing SAG induction by UCN and its peptides on primary neonatal cardiomyocytes. A. Cells were treated with 10^{-8} M of a peptide for 36 hours prior to protein harvest and analysed by western blotting. Actin was used as control. B. Densitometric analysis of at least three SAG blots. Columns represent the means and the bars the SD. Stars: MANOVA, $p<0.05$ vs control and $: MANOVA, $p<0.05$ vs all other homologues (Bonferroni post-hoc analysis).

7.2.2 SAG is inducible by hypoxia/reperfusion

Initially, we assessed the levels of SAG in cardiac cells exposed to 4 hrs of simulated ischaemia (Hypoxia) alone (H) or 4 hrs of simulated ischaemia plus 16 hrs of reoxygenation (H/R). As shown in Fig. 1, SAG protein levels were enhanced by 2.5 fold in primary neonatal cardiac myocytes exposed to 4 hrs of hypoxia alone compared to untreated control cells. The expression levels of SAG in neonatal cardiac myocytes following exposure to reoxygenation after hypoxia returned to control levels (Fig. 7.2).

We also assessed SAG expression in the isolated perfused rat heart that was exposed to either 35 mins of global ischaemia alone (I) or 35 mins of ischaemia followed by 60 mins of reperfusion (I/R). As shown in Fig. 7.3, the levels of SAG in the isolated intact heart exposed to 35 mins of ischaemia alone resulted in a 4.5 fold increase over the levels of SAG in the control (continuously perfused hearts). Additionally, as observed in neonatal cardiac myocytes, SAG levels dropped following reperfusion after ischaemia although they remained above control levels (Fig. 7.3). These results suggest that SAG expression in the heart is enhanced acutely by an ischaemic stress response, and that the increased levels fall during the reoxygenation/reperfusion phase.

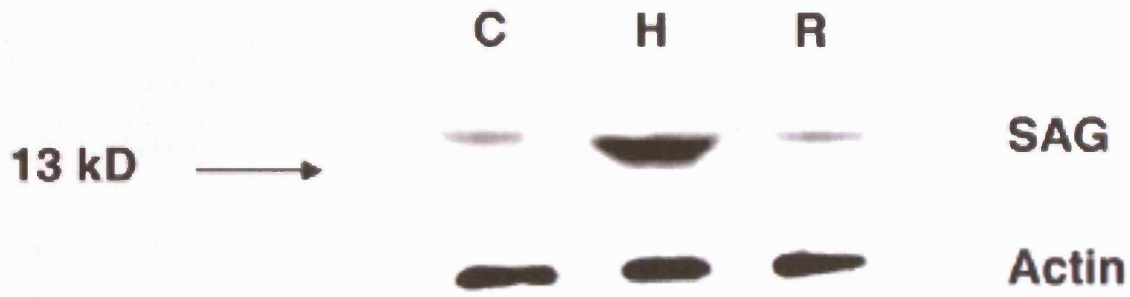


Figure 7.2. SAG is expressed in rat cardiomyocytes. Western Blot analysis with antibody to SAG of extracts from primary neonatal rat cardiomyocytes following 4 hours of hypoxia (H), or 4 hours of hypoxia followed by 16 hours reoxygenation (R), or no treatment (C). Actin is shown as a standard for the calibration of the SAG.

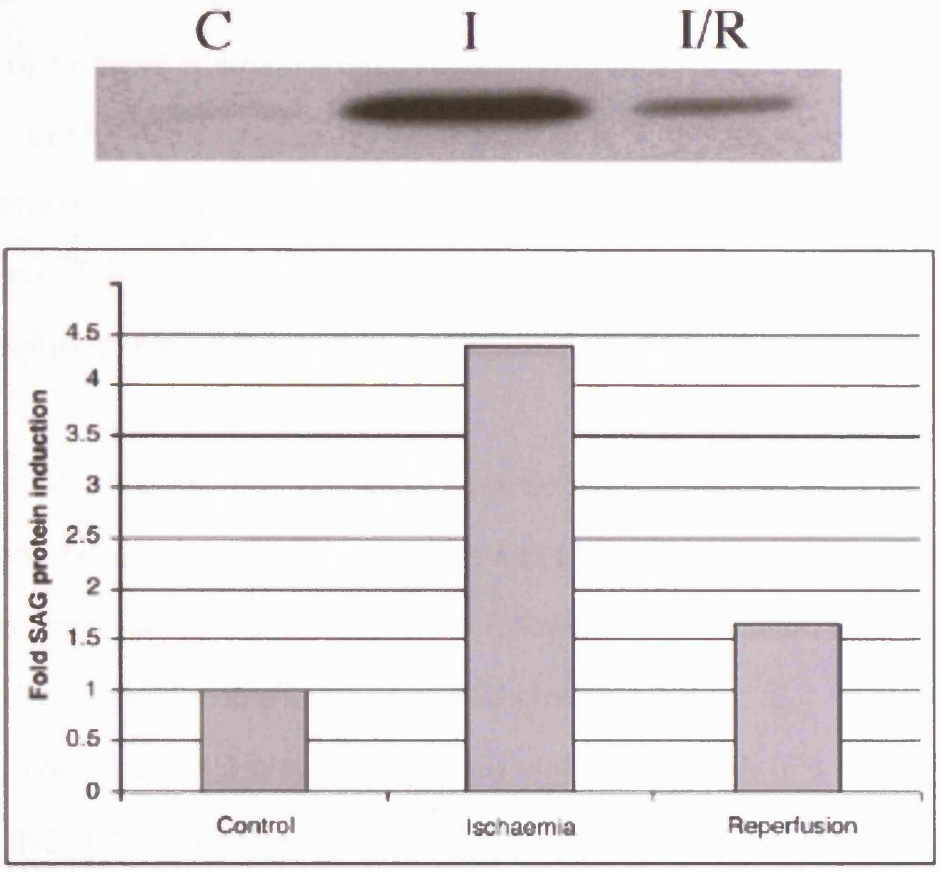


Figure 7.3. SAG is expressed in the intact rat heart. Western blot analysis with antibody to SAG of extracts from perfused rat hearts following 1 hour of global ischaemia (I) or 35 minutes of ischaemia followed by 1 hour reperfusion (I/R). The bar chart represents the Densitometric analysis of the above western blot.

In addition, immunocytochemical analysis of neonatal cardiomyocytes (Fig. 7.4A and B) revealed a strong expression in the cytoplasm and the nucleus of the cells. Confocal microscopy of those cells (Fig. 7.4B) revealed that the SAG expression in the nucleus appears to be restricted around the periphery of the nucleus. The pattern of localisation of SAG in neonatal cells appears to be maintained in the adult cells (Fig. 7.4C).

Since SAG has been described as an antioxidant factor, we were interested to explore the kinetics of SAG expression and to see whether the changes in SAG levels observed in cardiac myocytes following H/R are due to alterations in mRNA levels or due to alterations in SAG protein stability.

Time course of SAG protein (Fig. 7.5a) and SAG mRNA (Fig. 7.5b) induction reveals that the SAG protein level increased between 1 and 2 hours of simulated ischaemia (MANOVA $p<0.05$ vs. control) and reached a plateau after 2 hours (MANOVA $p>0.05$ between time points of 2 and 3 hours). Interestingly there was a steady increase of mRNA levels over the time period of the simulated ischaemic insult for the first four hour period (MANOVA $p<0.05$ for all time points vs. control).

In the case of reoxygenation following a 4 hour simulated ischaemia period, protein levels increased only slightly during the first hour of reoxygenation (MANOVA $p>0.05$), and were maintained for up to 4 hours (MANOVA $p<0.05$ for

all time points vs. control) (Fig. 7.6a). As before, SAG protein levels fell to almost that of the control cells after 16 hours of reoxygenation (Fig. 7.7).

Similarly, SAG mRNA levels were also slightly elevated for the first 4 hours of reoxygenation (MANOVA $p>0.05$ among the groups, but $p<0.05$ for all the groups vs. control) (Fig. 7.6b), only to return to control levels after 16 hours of reoxygenation. These results demonstrate that SAG expression involves enhanced mRNA levels leading to enhanced protein levels following a hypoxic signal. However, we cannot rule out that posttranslational events may also be contributing to SAG upregulation during exposure of cardiac myocytes to H/R. As SAG levels were increasing with increasing times of simulated ischaemia we were interested to see if the levels of SAG protein might be different following reoxygenation after different periods of simulated ischaemia. Surprisingly, SAG protein levels were always similar to control after 16 hours of reoxygenation (MANOVA $p>0.05$ for all groups at reoxygenation vs. control), regardless of the duration of simulated ischaemia, where SAG protein levels increased over time, suggesting a very tight control of SAG protein levels (Fig. 7.7). Moreover, this suggests that SAG has an important role during periods of low oxygen supply to the cell.

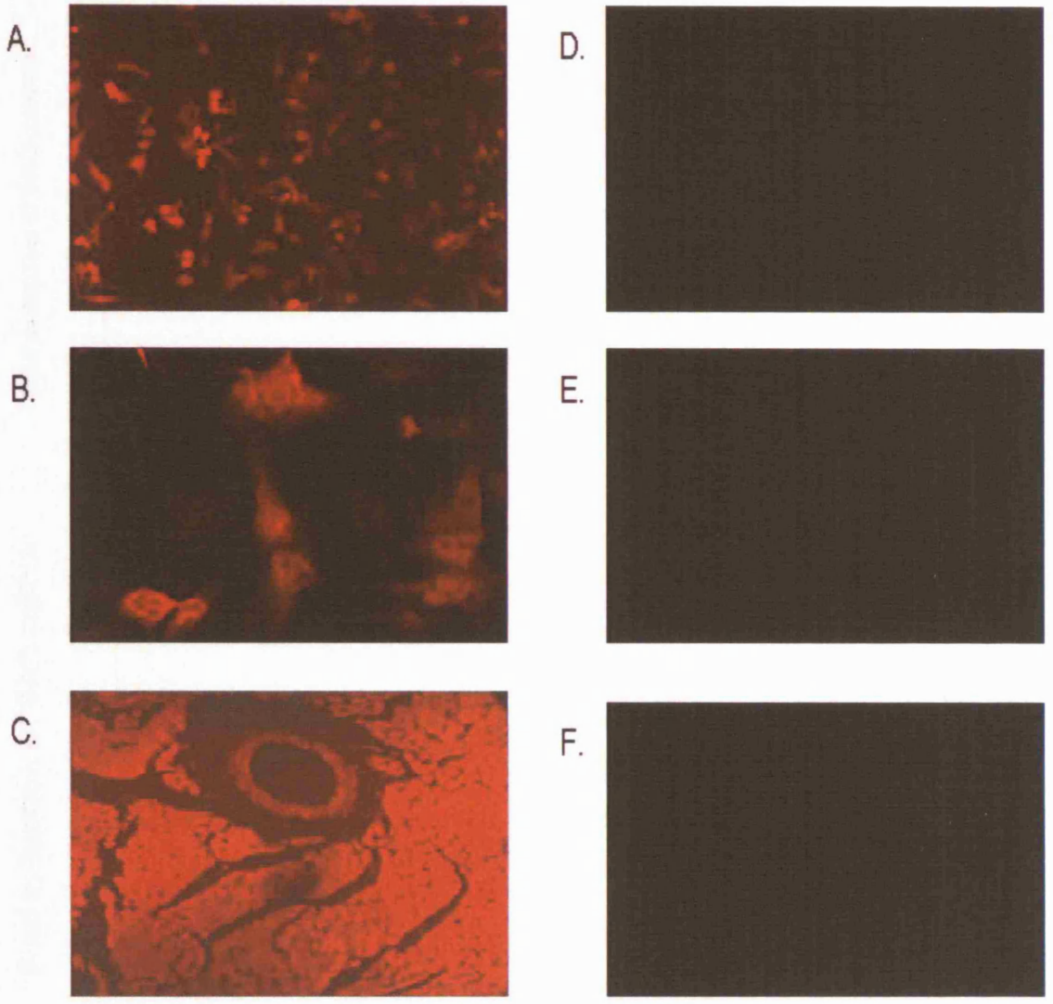


Figure 7.4. SAG is expressed in heart cells. SAG is expressed in primary neonatal cardiomyocytes (A and B) and in adult heart (C). Immunocytochemistry (A and C) reveals a nuclear and cytoplasmic localisation of SAG in cardiomyocytes. Negative reactions (no secondary antibody) are shown in the right column.

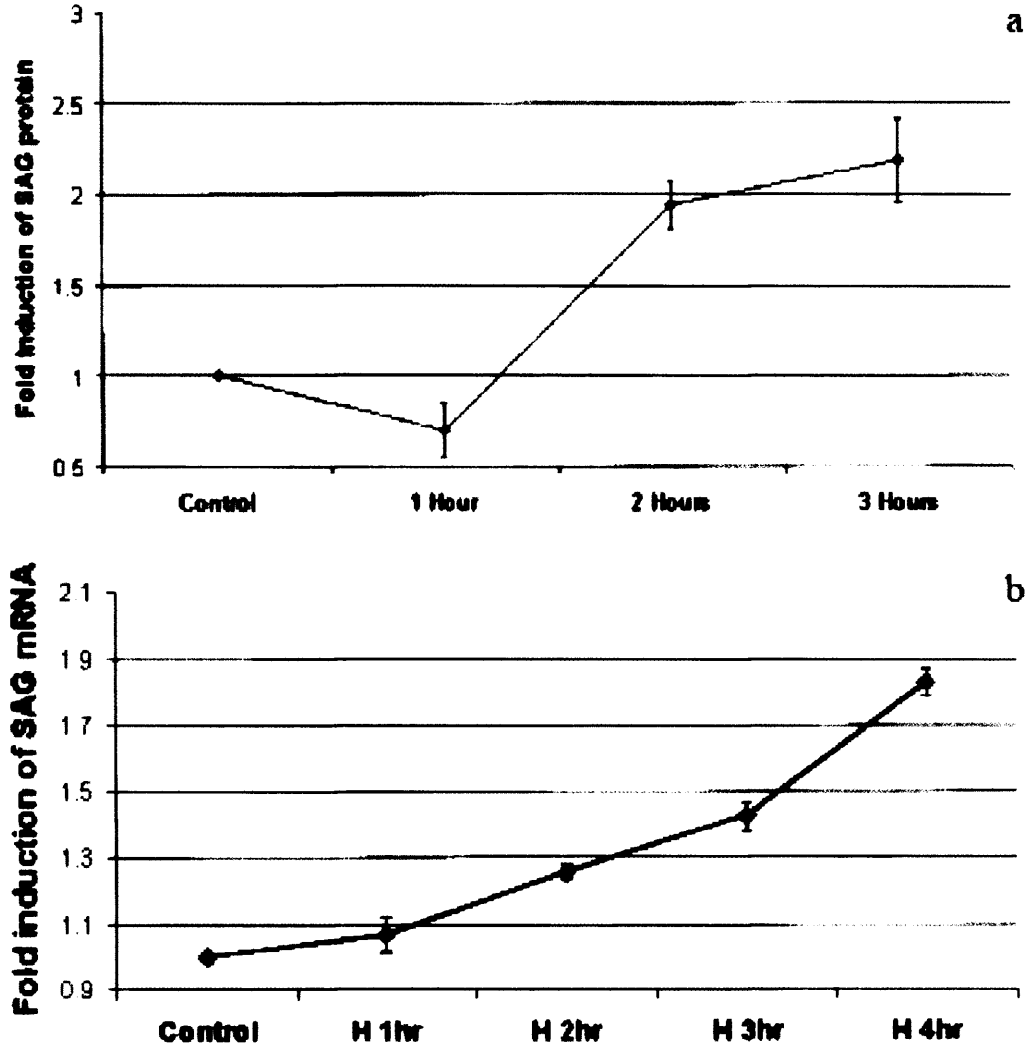


Figure 7.5. SAG is induced by hypoxia in cardiomyocytes. Densitometric analysis of SAG protein (A) and mRNA (B) temporal kinetics following different periods of simulated ischaemia. Bars represent standard deviations of at least three independent experiments. Western blotting for actin (A) and RT-PCR for actin was used as a standard for the calibration of the amounts of SAG in each case.

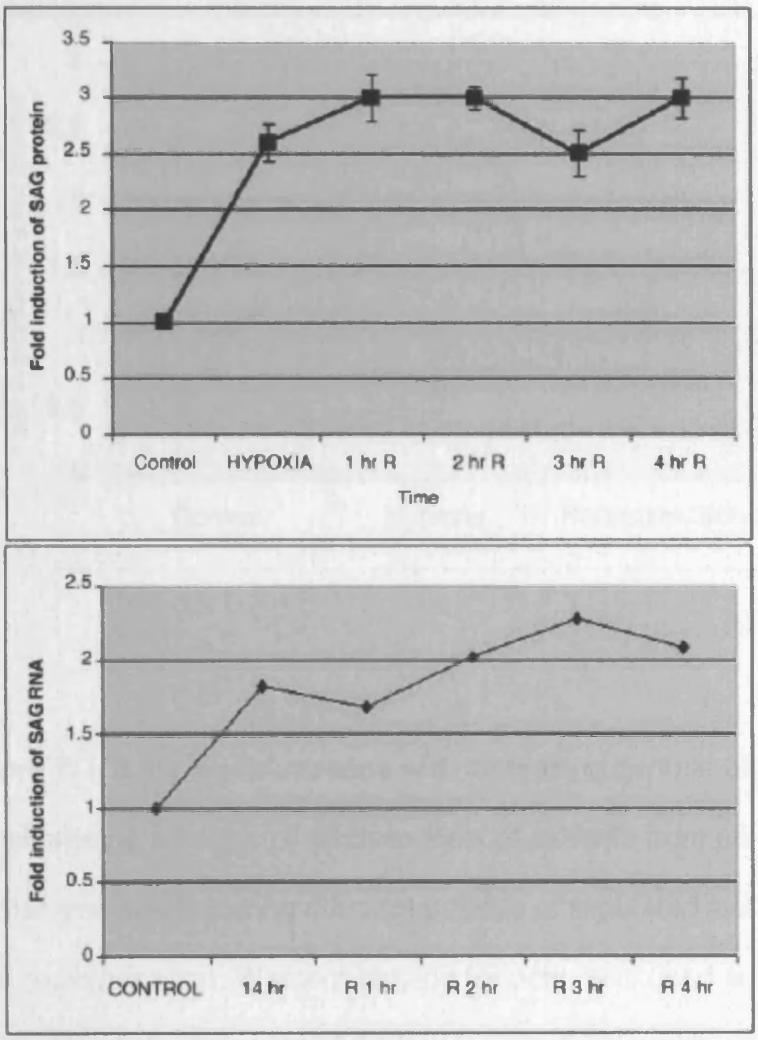


Figure 7.6. SAG levels remain elevated for the first four hours of reoxygenation. Densitometric analysis of SAG protein (A) and mRNA (B) temporal kinetics during different periods of reoxygenation, following 4 hours of simulated ischaemia. Bars represent standard deviations of at least three independent experiments. Western blotting for actin (A) and RT-PCR for actin was used as a standard for the calibration of the amounts of SAG in each case.

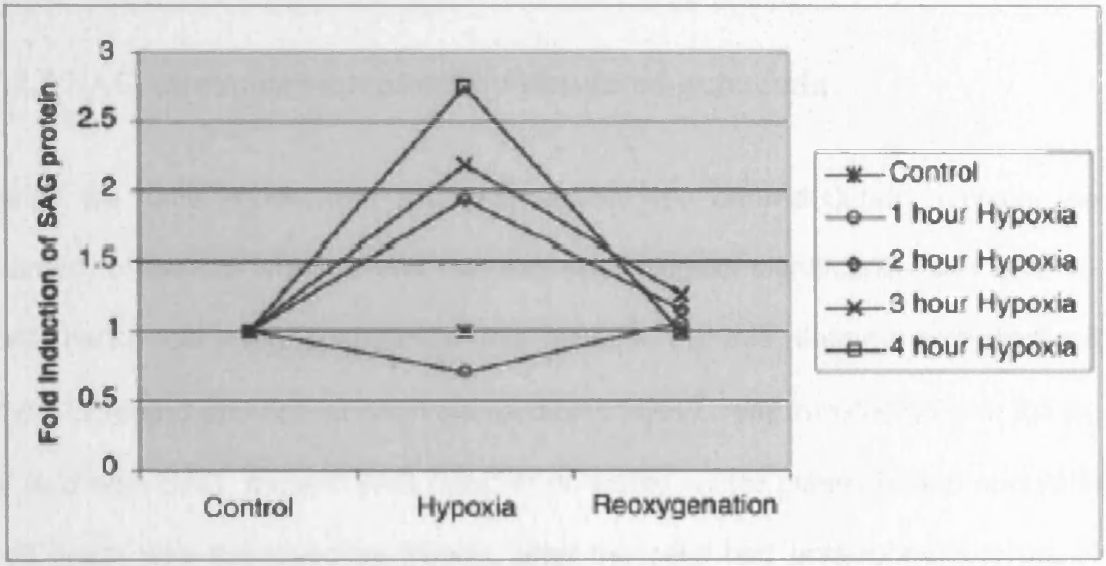


Figure 7.7. SAG levels increase with increasing periods of hypoxia. Densitometric analysis of western blots of extracts from primary neonatal rat cardiomyocytes following different periods of simulated ischaemia followed by 16 hour reoxygenation. Western blotting for actin was used as a standard for the calibration of the amounts of SAG.

7.2.3 SAG attenuates apoptosis by simulated ischaemia

Since we have shown that SAG expression was altered during hypoxia, we wanted to find out whether that had any physiological significance and whether SAG had a role in protecting cells from hypoxia. For this reason the cardiac cell line CLEM and primary rat neonatal cardiomyocytes were transfected with 2.5 μg of wild type SAG, mutant SAG (MM14) or empty vector plasmids and apoptotic cell death was assessed by TUNEL after the cells had undergone 4 hours of simulated ischaemia followed by 16 hours of reoxygenation. The mutant SAG MM14 has two cysteines in the RING finger mutated to serines. This mutation has been previously shown to render the SAG protein non-functional. To discriminate between transfected and non-transfected cells the beta-galactosidase plasmid was used as a marker of transfected cells.

Overexpression of SAG was able to reduce the amount of apoptosis after H/R almost to the control levels not only in CLEM cells (MANOVA $p>0.05$ vs. normoxia control group) (Fig. 7.8) but also in primary cardiomyocytes (MANOVA $p>0.05$ vs. normoxia control group) (Fig. 7.9). TUNEL positivity in SAG transfected CLEM cells after H/R was 50% of that in cells transfected with the empty vector (MANOVA $p<0.05$ vs. control group). The antiapoptotic effects of SAG after reoxygenation was even more pronounced in the primary neonatal rat cardiomyocytes, where the TUNEL positive SAG transfected cells were almost

60% of the cells transfected with the empty vector (MANOVA $p<0.05$ vs. control). Interestingly, SAG was able to protect cells from apoptotic death due to simulated ischaemia alone (MANOVA $p>0.05$ vs. control), as well as after reoxygenation (MANOVA $p>0.05$ vs. control).

In addition, the apoptotic protection observed by SAG was dependent on a functional RING finger, as transfection of MM14 a mutant that has a dysfunctional RING finger, attenuated the protective effects of SAG, both in CLEM cells (Fig. 7.8) and primary rat cardiomyocytes (Fig. 7.9). The levels of protection in the CLEM cells transfected with MM14 was similar to those of CLEM cells transfected with the empty vector not only after H (MANOVA, $p>0.05$ vs. control hypoxia) but also after H/R (MANOVA, $p>0.05$ vs. control reoxygenation) (Fig. 7.8). Similarly transfection of MM14 in primary rat cardiomyocytes had no effect on the levels of protection after H (MANOVA, $p>0.05$ vs. control hypoxia) and also after H/R (MANOVA, $p>0.05$ vs. control reoxygenation) (Fig. 7.9). These results clearly demonstrate the importance of the integrity of the RING finger domain of SAG for the antiapoptotic function of the protein.

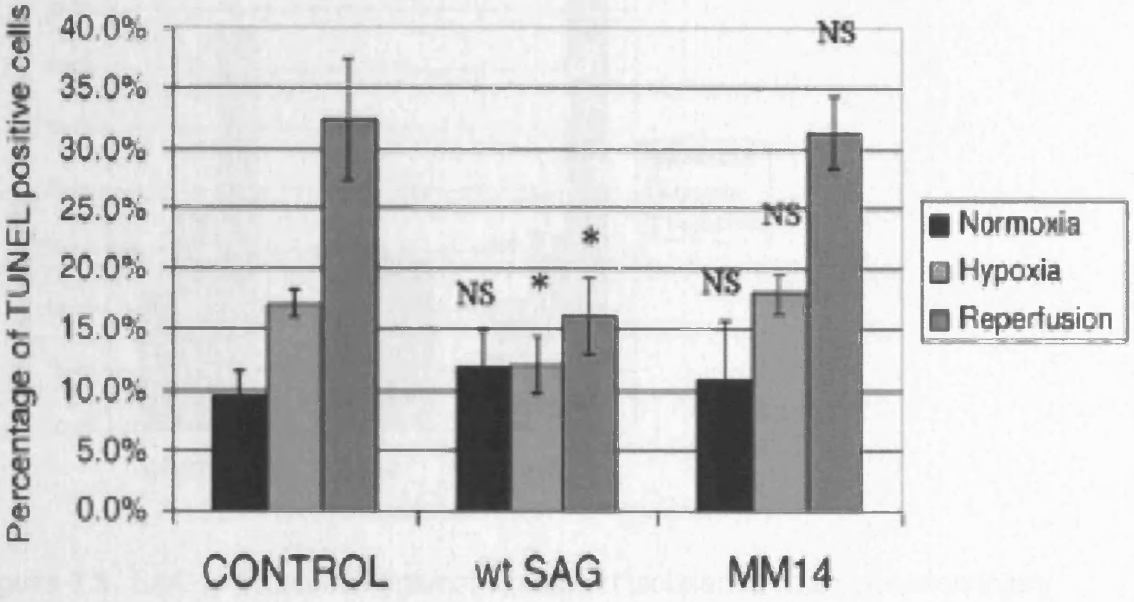


Figure 7.8. SAG is protective against simulated ischaemia reoxygenation injury in CLEM cells. 5μg of total DNA was transfected in CLEM cells, by the calcium phosphate method as described in Materials and Methods, prior to 4 hours of hypoxia or 4 hours of hypoxia followed by 16 hour reoxygenation. MM14 is a mutant of SAG that has a non-functional RING finger domain. Percentage of TUNEL positive transfected CLEM cells are represented by the columns. Values are the average of three experiments, whose standard deviations are represented by the bars. Stars represents statistical significance against control (MANOVA, $p<0.05$). NS represents no difference against control.

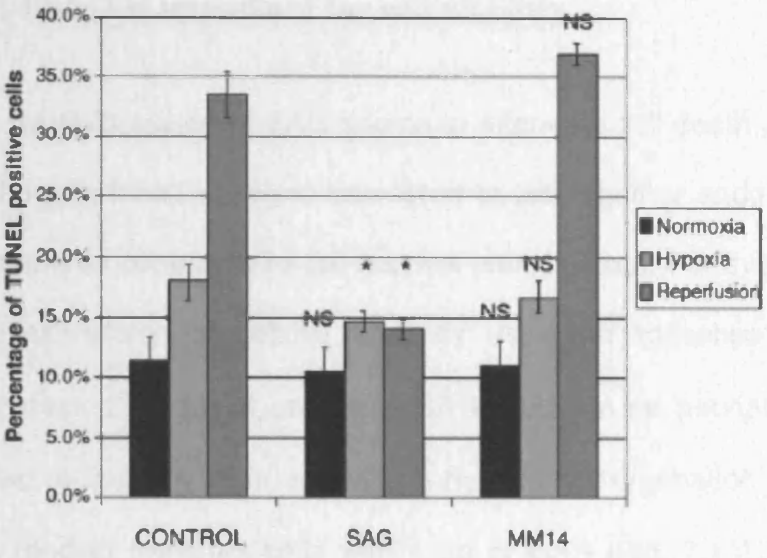


Figure 7.9. SAG is protective against simulated ischaemia reoxygenation injury in rat neonatal cardiomyocytes. 5μg of total DNA was transfected in primary rat neonatal cardiomyocytes, by the calcium phosphate method as described in Materials and Methods, prior to 4 hours of simulated ischaemia and 4 hour of simulated ischaemia followed by 16 hour reoxygenation. Percentage of TUNEL positive transfected rat neonatal cardiomyocytes are represented by the columns. Values are the average of three experiments, whose standard deviations are represented by the bars. Stars represents statistical significance against control (MANOVA, $p<0.05$). NS represents no difference against control.

7.2.4 SAG is important for cell viability

As overexpression of SAG seems to attenuate cell death caused by a simulated ischaemic insult we were interested to test whether endogenous levels of SAG are able to contribute to cell survival after hypoxia. For this reason we abrogated the expression of cellular SAG by using an antisense approach. When we transfected 2.5 μg of antisense SAG (GAS) in rat neonatal cardiomyocytes we failed to see any cells surviving a hypoxia reoxygenation injury. For this reason we tried to transfect cells with 1 μg of GAS (Fig. 7.10). In this case although overexpression of SAG again led to attenuation of apoptotic cell death after 4 hours of simulated ischaemia followed by 16 hour reoxygenation (MANOVA, $p>0.05$ vs. control reoxygenation), GAS overexpression led to an increase of apoptotic death following reoxygenation compared to control cells (MANOVA, $p<0.05$ vs. control reoxygenation) (Fig. 9). Moreover, we observed that the levels of apoptotic death in cells transfected with the antisense SAG were greatly increased even in the absence of hypoxia/reoxygenation (MANOVA, $p<0.05$ vs. control normoxia), indicating that SAG is essential for the survival of cultured neonatal rat cardiomyocytes.

The above results show that SAG is essential for the survival of cardiomyocytes not only after an ischaemic insult, but also under normal, non stressful conditions.

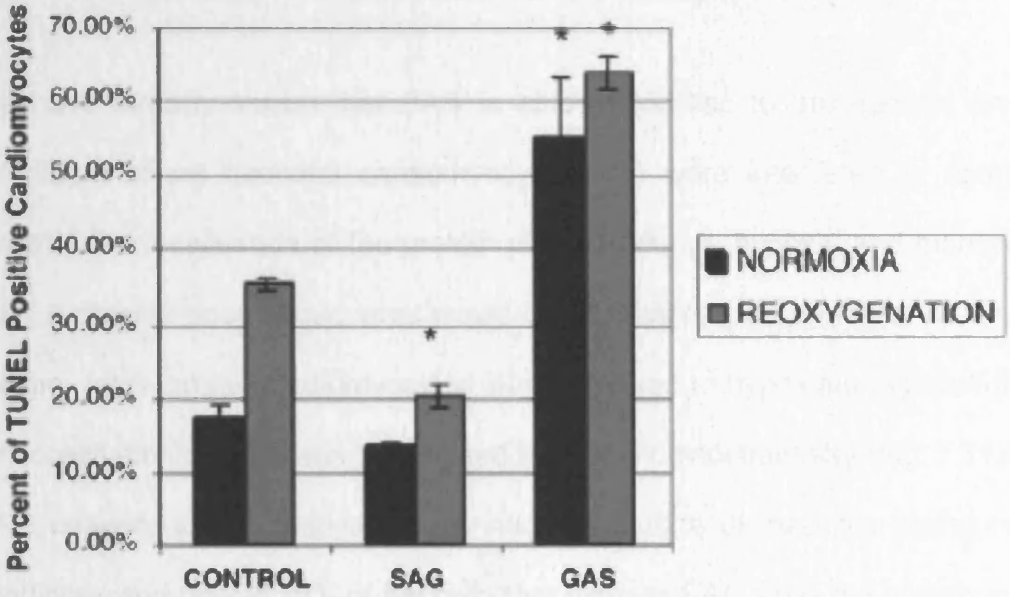


Figure 7.10. SAG expression is essential for the viability of neonatal cardiomyocytes under hypoxic and normoxic conditions. Overexpression of antisense SAG (GAS) is lethal for primary neonatal rat cardiomyocytes. 2μg of total DNA was transfected in primary rat neonatal cardiomyocytes, using lipofectamine 2000 as described in the Materials and Methods, prior to 4 hour of hypoxia followed by 16 hour reoxygenation. Percentage of TUNEL positive transfected rat neonatal cardiomyocytes are represented by the columns. Values are the average of three experiments, whose standard deviations are represented by the bars. Stars represents statistical significance against control (MANOVA, $p<0.05$). NS represents no difference against control.

7.2.5 SAG localisation is important for its antiapoptotic properties.

We have already shown that SAG is able to localise to the nucleus and the cytoplasm of rat neonatal cardiomyocytes. We were interested to determine whether the localisation of the protein changes during hypoxia and moreover, if such a change does occur, what would be its physiological significance. For this reason, rat neonatal cardiomyocytes were exposed to hypoxia/roxygenation and the localisation of SAG was determined by immunocytochemistry (Fig. 7.11).

SAG appears to be localised to the nucleus in 82% of the cells during control conditions and only in 18% of the cells that express SAG does the protein appear exclusively in the cytoplasm (Fig. 7.11). This pattern of expression however changes after hypoxia reoxygenation, where even though the pattern of expression appears to be predominantly nuclear there is an increase in the cytoplasmic SAG (Fig. 7.11).

To examine further the role of cytoplasmic SAG we analysed the amount of apoptosis in cells stained for SAG by TUNEL. Interestingly only 20% of the cells expressing SAG were TUNEL positive after hypoxia reoxygenation (Fig. 7.12). When we looked at the SAG localisation only 10% of the cells that were expressing SAG in the cytoplasm were TUNEL positive compared to almost 40% apoptotic cells that were expressing SAG in the nucleus. Only 25% of the cells that were expressing SAG in both cell compartments were TUNEL positive. From the above we conclude that SAG localisation in the cell is important for cell

viability. It appears that only when SAG is expressed in the cytoplasm of the cell is the protein able to bring about its antiapoptotic effects.

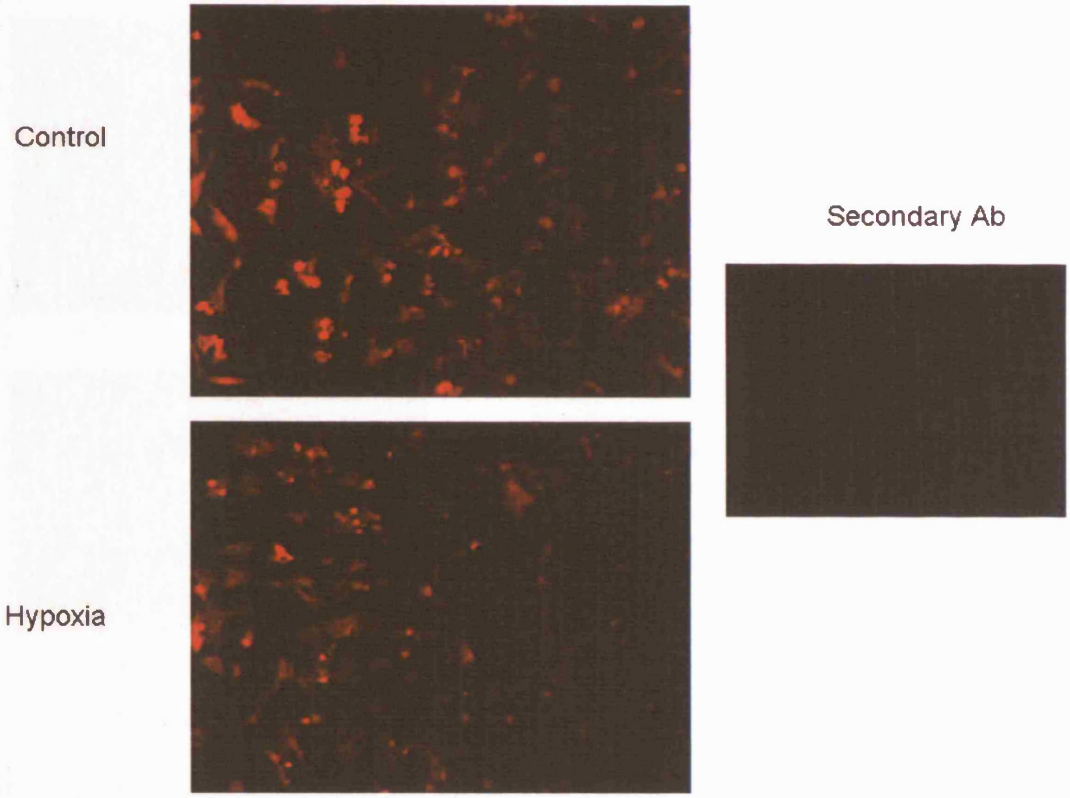
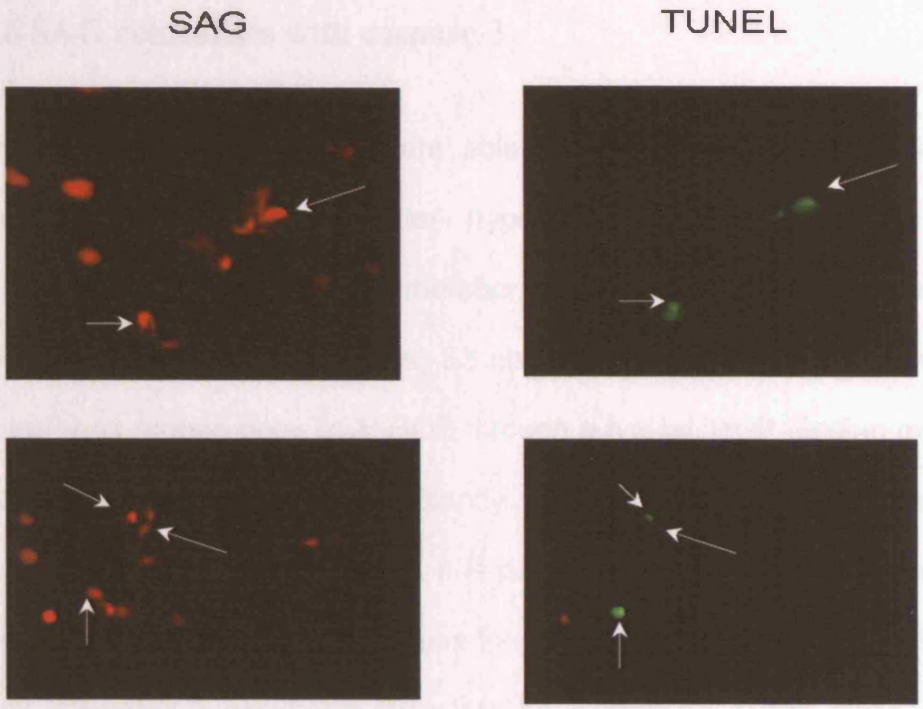


Figure 7.11. Immuno-localisation of SAG in control rat neonatal cardiomyocytes and in cells undergone 4 hours of hypoxia and 6 hours of reoxygenation.



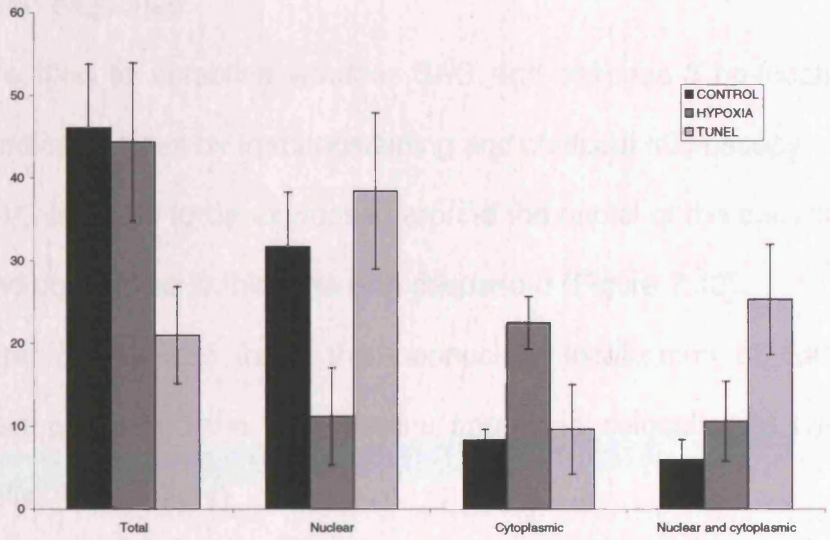


Figure 7.12. SAG staining (red) on TUNEL stained (green) primary neonatal cardiomyocytes undergone 4 hour hypoxia and 4 hour reperfusion.
The graph shows the percentage of cells expressing SAG in the nucleus or the cytoplasm of the cell. Black bars represent percentage of cells expressing SAG under control conditions, dark grey: SAG positive after hypoxia and light gray: TUNEL positive and SAG positive cells

7.2.6 SAG colocalises with caspase-3

From the previous results we are able to suggest that cytoplasmic SAG is important for cell viability after hypoxia reoxygenation in rat neonatal cardiomycytes. SAG or its family member, ROC1/Rbx1 is an active component of SCF (Skp1-Cullins-F-box proteins) E3 ubiquitin ligase. SAG binds to caspase 3 via an F-box protein beta-TrCP/HOS through a typical TrCP binding motif and b-TrCP/HOS over-expression significantly shortens protein half life of caspase 3 (Sun Yi, personal communication). It is possible that SAG as part of the SCF E3 needs to translocate to the cytoplasm in order to keep the levels of caspase-3 in check. Caspase-3 has been shown to be expressed in the nucleus and the cytoplasm of cells (Loo et al., 2002), but its localization in cardiac cells has not been examined.

We tried to establish whether SAG and caspase-3 co-localise in rat neonatal cardiomyocytes by immunostaining and confocal microscopy.

SAG appears to be expressed around the nuclei of the cells in control conditions and co-localise in this area with caspase-3 (Figure 7.13).

After an hypoxic insult this perinuclear localization of SAG and Caspase-3 disappears and the two proteins appear to colocalise in the cytoplasm of the cells. (Figure 7.14).

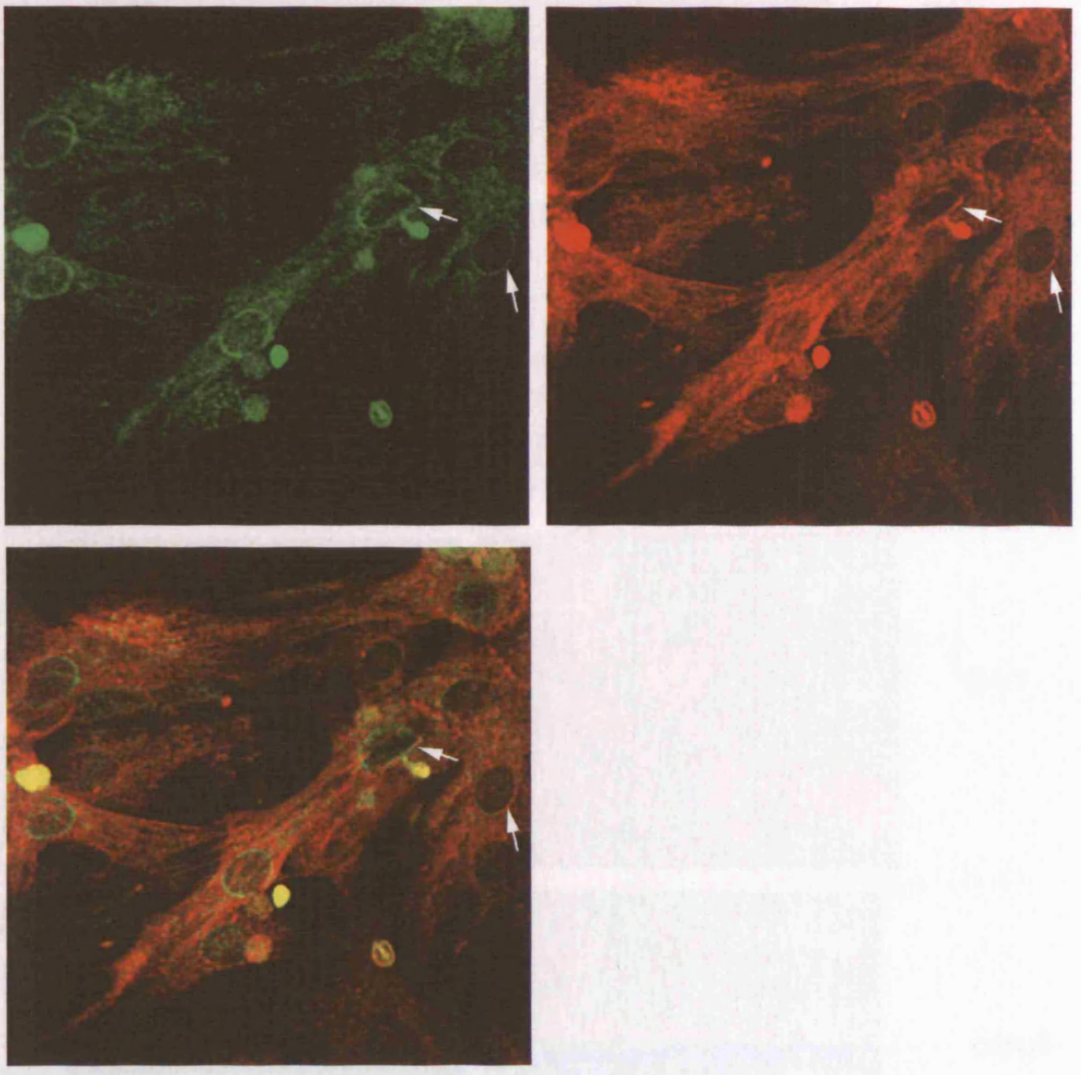


Figure 7.13. Rat neonatal cardiomyocytes stained with SAG (red) and caspase-3 (green). Overlap of the two pictures shows SAG and caspase-3 colocalisation (yellow-brown) around the nucleus and in the cytoplasm.

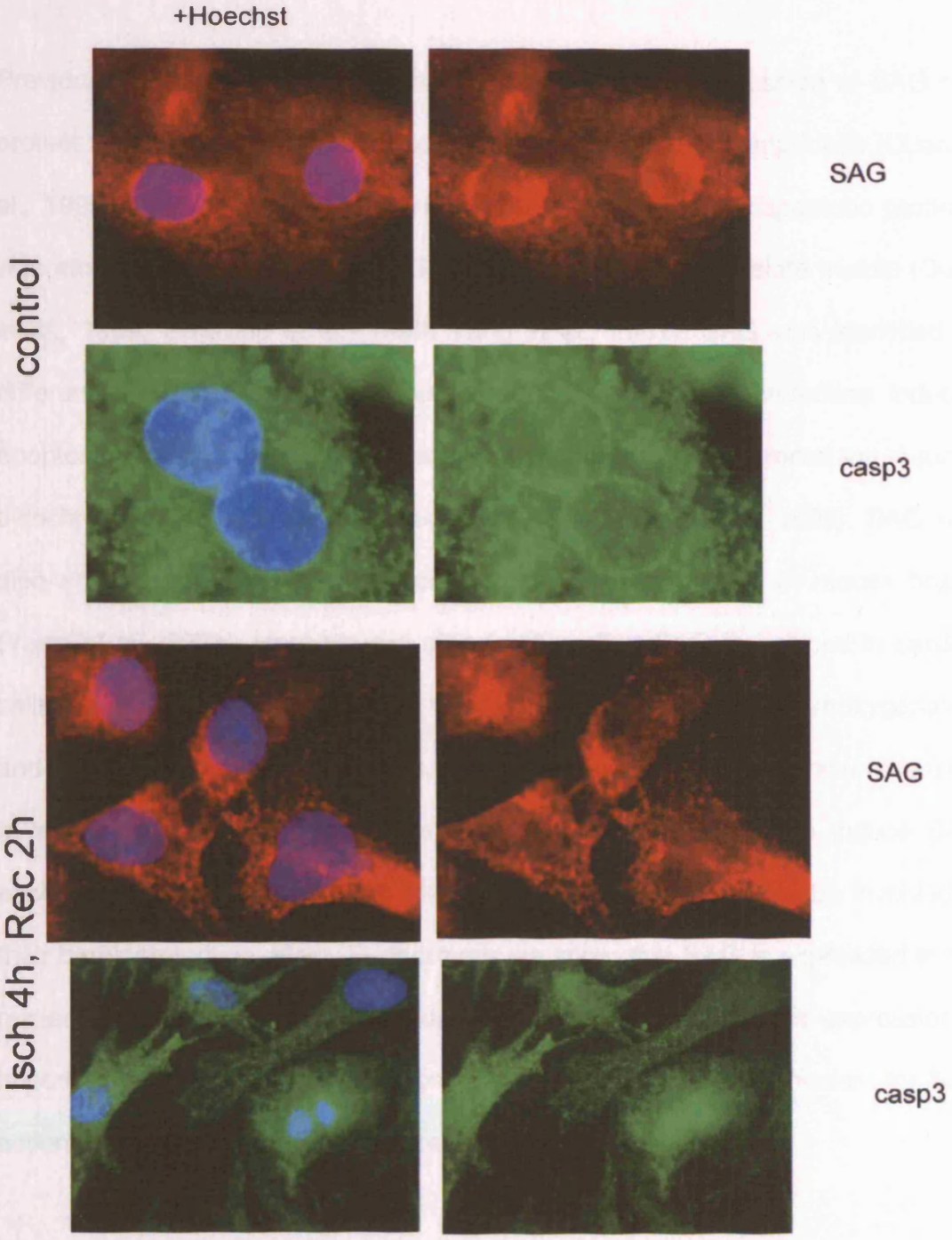


Figure 7.14. Rat neonatal cardiomyocytes were stained with Hoechst (blue), SAG (red) and caspase-3 (green) and analysed by confocal microscopy.

7.3 Discussion

Previous work in non cardiac cells has shown that overexpression of SAG can protect non cardiac cells from apoptosis induced by redox compounds (Duan et al., 1999; Swaroop et al., 1999; Yang et al., 2001). This antiapoptotic property was attributed to the ability of SAG to scavenge ROS and chelate metals (Duan et al., 1999; Swaroop et al., 1999; Yang et al., 2001). SAG was identified by differential display as a gene that responds to 1,10-phenanthroline induced apoptosis (Duan et al., 1999). It was shown to inhibit or delay metal ion induced cytochrome c release and caspase 7 activation (Duan et al., 1999). SAG was also shown to protect against ischaemic induced apoptosis in mouse brains (Yang et al., 2001). Here we are able to show that SAG is induced in cardiac cells and in the intact heart during hypoxic stress and not during reoxygenation and that it protects cardiomyocytes from the damaging effects of these stresses. In addition, we show that UCN and its homologues is able to induce SAG expression in rat neonatal cardiomycytes and this may therefore be involved in their cardioprotective effect. Furthermore we show that SAG is expressed in the nucleus and the cytoplasm of the cells and that SAG cytoplasmic expression is important for cell viability. We go on to propose a possible mechanism for SAG action by possible interaction with caspase-3.

Interestingly, the levels of SAG rise during ischaemia/hypoxia in both cultured cells and in the intact heart, but decrease to control levels during subsequent

reoxygenation/reperfusion. This suggests that SAG expression is induced specifically due to processes occurring during hypoxia/ischaemia, such as lowered oxygen levels and returns to normal as oxygen levels are restored during reoxygenation/reperfusion. The fact that SAG is upregulated during ischaemia might also suggest that it is involved in protecting cardiomyocytes from the incoming increase of ROS during reperfusion and acts as a first defensive mechanism for the cell.

As in other cell types, overexpression of SAG is able to protect primary cultured neonatal rat cardiomyocytes from apoptosis induced by simulated ischaemia/reoxygenation and also protects the intact heart from ischaemic damage. Moreover, we show for the first time that endogenous SAG expression is important for the viability of neonatal rat cardiomyocytes in the absence of stress.

The protection observed following overexpression of SAG is specific, as overexpression of the mutant MM14 with an abrogated RING finger (Swaroop et al., 1999), failed to show any protection. This result is in agreement with previous data in other cell types (Sun, 1999; Swaroop et al., 1999; Yang et al., 2001) and confirms the importance of the RING finger domain for the function of SAG. RING finger domains have been described to be important for protein-protein interaction and most importantly in mediating protein ubiquitination (Joazeiro and Weissman, 2000; Tyers and Jorgensen, 2000). This suggests that SAG may also

bring about its antiapoptotic properties by targeting specific proteins for proteosomal degradation.

SAG belongs to a family of proteins that includes ROC1/Hrt1/Rbx1. SAG and ROC1 are evolutionary and functionally conserved as mammalian ROC1 and ROC2 can rescue ROC1 deficient yeast cells (Ohta et al., 1999). The RING domain of ROC1 has been shown to be essential for ubiquitin ligation (Chen et al., 2000). SAG has recently been implicated in regulating p27 in mammalian cells and promotes S-phase entry and cell growth under serum starvation (Duan et al., 2001). It is possible that SAG may also be regulating p27 levels in cardiomyocytes. Interestingly, the Insulin like Growth Factor I (IGFI) was shown to elicit its antiapoptotic effects in rat cardiomyocytes by downregulation of p27 and p21 (Von Harsdorff et al., 1999). P27 belongs to the kinase inhibitor protein family of cell cycle inhibitor proteins that regulate cell cycle and promote cell cycle arrest. Such a model would explain the elevated cell death when we overexpressed GAS (anti-sense SAG) in cardiomyocytes, as upregulation of p27 has been shown to be a step leading to apoptosis in a number of cell types (Von harsdorff et al., 1999; Donjerkovic et al., 2000; Li et al., 2000; Nakatsuji et al., 2001).

Protection by SAG appears to be related to its location in the cell. We have shown that cytoplasmic SAG is translocated to the cytoplasm after hypoxia and that the cytoplasmic SAG is likely to be responsible for the antiapoptotic

properties of SAG. It appears that SAG needs to regulate the levels of a specific protein or proteins in the cytoplasm for cell protection, probably as part of an SCF E3 ligase. This explains the importance of the RING finger in our mutant overexpression experiments. SAG is an active component of SCF (Skp1-Cullins-F-box proteins) E3 ubiquitin ligase. Beta-TrCP/HOS over-expression has been shown to significantly shorten the protein half life of caspase 3 (Sun Yi, personal communication). It is possible that SAG as part of the beta-TrCP/HOS containing SCF E3 needs to translocate to the cytoplasm in order to keep the levels of caspase-3 in check. Our results show a possibility for a SAG-caspase-3 colocalisation in the heart cells. However, we lack evidence as yet for a direct interaction between the two proteins or through a third protein.

SAG is undoubtedly a protein that has an important function in the heart. Its expression increases after hypoxic conditions and it has a protective function in cardiomyocytes both prior to stress and when exposed to ischaemia/hypoxia. It also appears to be playing a role in UCN mediated cardioprotection.

It is important to identify the interactions of SAG in the heart and the mechanism of function of this protein, as its distinct anti-ischaemic properties indicate that it may have therapeutic potential in ischaemia and failing hearts.

CHAPTER VIII

General Discussion

In this work we set out to examine the effects of urocortin and its homologues in the rat heart, after hypoxia/reoxygenation injury in order to determine possible differences in the level of cardioprotection conferred by the different peptides. In addition, we looked into the pathways that are activated by UCN and its homologues and their involvement in cardioprotection. Furthermore, the effects of UCN and its homologues on cardiac hypertrophy were examined and we present at least one of the pathways required for the hypertrophic effects of UCN. We selected the rat neonatal primary cardiomyocytes as our model for our experimentations, as they are easily isolated, retain cardiac contractility and are adequately transfected with exogenous plasmids, in order to study the effects of certain genes in the cardiac physiology and their involvement in the UCN action. In addition, a few experiments were performed in an adult rat heart to compliment some of the results obtained from the primary cells.

We started our studies by showing that UCN, SCP and SRP are all present and expressed in the rat primary neonatal cardiomyocytes.

We showed for the first time that SRP in control conditions is expressed at very low levels. On the contrary, it was previously shown that SCP mRNA in the adult mouse and human heart are expressed at low levels (Hsu and Hsueh, 2001; Lewis et al., 2001). These results were in contrast to our conclusions. A possible explanation could be that SCP and SRP levels change during heart

development, or that SCP and SRP follow different patterns of expression in the rat due to species specific factors. Clearly, more work is needed in that part to determine why this is so. Interestingly enough, after a hypoxic stress, the levels of all three peptides in the rat neonatal cardiomyocyte were comparable.

The possibility that SCP or SRP are only expressed in certain species after a certain stress has occurred raises an interesting question that needs to be further examined. Could there be an evolutionary trend emerging, where the stress coping peptides evolving in order to be expressed only when required? That should certainly make sense as it could explain the specificity of the SCP and SRP for the CRFR2 and the presence of multiple CRF homologous peptides that is observed in the mammals. A study is needed to show the pattern of expression of the SCP and SRP peptides in higher mammals and especially primates. Also, if such a pattern occurs, then there must be differences in the promoter region of CRF and UCN compared to the SCP and SRP that could shed light in the factors that drive the expression of the SCP and SRP genes and these factors will probably emerge as key players in orchestrating the stress response at the molecular level.

Having established that all three peptides are expressed in the neonatal heart, we set out to determine their cardioprotective effects. We compared the effects of all three peptides in protecting neonatal cardiomyocytes after hypoxia alone, or after hypoxia/reoxygenation injury against total cell death as measured by Trypan

blue exclusion. We showed that all three peptides minimised the levels of trypan blue exclusion when given either before or after hypoxia and no differences in the levels of protection were observed between the three peptides when used at a concentration of 10^{-8} M.

This result was somewhat surprising, as our original hypothesis was that because the three peptides show different affinities for the CRFR2b the cardioprotective effects of the peptides would resemble their affinities to the receptor. So, in order to further examine our hypothesis, we compared the effects of the three peptides against apoptosis using a series of concentrations. This time we were able to show that SCP and SRP were more protective than UCN when used at a concentration of 1pM, when added prior to hypoxia/reoxygenation and more protective than UCN at concentrations of 1 and 10 pM when used prior to the onset of reoxygenation. Furthermore, SCP was more protective than SRP at a concentration of 1pM when used prior to reoxygenation, even though that SRP exhibits higher affinity to the CRF2b receptor than SCP (Hsu and Hsueh, 2001).

This could be due to a possible lower affinity of the human SRP to the rat receptor or because of the higher ability of UCNIII/SCP to activate the receptor CRFR2β compared to UCNII/SRP, as measured by accumulation of cAMP (Lewis et al., 2001). This could also explain the similar protective properties of

UCN to the two homologues, as the relative potencies of SCP, SRP and rat UCN to functionally activate the receptor, overlap (Lewis et al., 2001).

Another reason for the differences in the protective effects of the SCP and SRP at the 1pM level, could be due to the lack of C-amidation of the SRP peptide we used. Amidation of the CRF peptides has been proposed to be important for their biological activity. It is essential for peptide maturation and it might be required for correct protein folding or for additional places for receptor-peptide interaction. The lack of SRP amidation could explain the slightly lower biological activity of the peptide compared to SCP.

UCN has been shown to require the activation of MAPK p42/44 and Akt/PKB in order to elicit its protective effects in the rat heart and in isolated cardiomyocytes (Brar et al., 2000; Latchman, 2001; Brar et al., 2002a; Brar et al. 2002b; Dautzenberg and Hauger, 2002; Railson et al., 2002; Schulman et al., 2002). Here we are able for the first time to show that SCP and SRP require activation of p42/44 MAPK and Akt to bring about their antiapoptotic functions in the primary rat neonatal cardiomyocytes (Fig. 5.2).

Moreover, we show that blocking phosphorylation of Akt and p42/44 MAPK either by chemical inhibitors, or by dominant negative constructs, we can increase the amount of cell death following hypoxia reoxygenation (Fig. 5.2), in accordance with previous results (Yue et al., 2000; Yamashita et al., 2001) and extended to

show that SCP and SRP work in a similar fashion indicating a common pattern of cardioprotection mechanism among the UCN homologues. Moreover, blocking of p38 MAPK can improve viability following hypoxic insults (Fig. 5.2), as shown before (Ma et al., 1999; Mackay et al., 1999; Yue et al., 2000).

Furthermore, we are able to demonstrate that SCP is a more potent activator of the p42/44 MAPK, followed by UCN and SRP last (Fig. 5.1). This pronounced activation of p42/44 MAPK by SCP can also explain the potent cardioprotective effects of SCP, and can further strengthen the notion that SCP is binding to CRFR2 with higher affinity than UCN. It has been previously shown in myometrial cells that phosphorylation of the MAPK p42/44 is a downstream event that follows the activation of CRFR1α and CRFR2β (Grammatopoulos et al., 2000).

Our results demonstrate that UCN homologues require activation of both p42/44 MAPK and Akt, in order to protect cardiomyocytes from hypoxia/reoxygenation injury (Fig. 5.1, 5.2). This would suggest that the two pathways might be linked. We proposed that ras could be one of the signalling molecules induced by PKA. This model could explain the increase in p42/44 phosphorylation observed after treatment with SCP and UCN in the presence of LY294002 (Fig. 5.1). However this had no effect in protection (Fig. 5.2), suggesting that UCN peptides need activation of downstream protective effectors regulated by both pathways.

Finally, inhibition of p38 MAPK increased phosphorylation of p42/44 MAPK (Fig. 5.1), in the presence of UCN and SCP. Again, this would suggest a crosstalk between the different pathways. This crosstalk between p38 MAPK and p42/44 MAPK has been shown to occur in epithelial corneal cells, where chemical inhibition of p38 MAPK increased phosphorylation of p42/44 MAPK in the presence of hepatocyte growth factor (Sharma et al., 2003).

UCN and its homologues have attracted great attention because of their cardioprotective effects as possible pharmacological agents on patients with ischaemic heart disease. However, UCN has also been shown to be involved in cardiac hypertrophy. UCN is able to increase distinct markers of hypertrophy, such as protein synthesis, cell size, atrial (ANP) and brain (BNP) natriuretic peptide secretion and collagen in cardiomyocytes (Ikeda et al., 1998; Nishikimi et al., 2000; Railson et al., 2002). For this reason we set out to explore the hypertrophic effects of the three peptides on rat neonatal cardiomyocytes.

Hypertrophy is characterised by an increase in cell size, increase in protein synthesis and activation of specific genes. For these reasons we set out to show the effects of the three peptides on all three of the above markers of hypertrophy. All three peptides increased cell size, protein to DNA ratio and the levels of mRNA of ANP and BNP when used at 10^{-8}M for 48 hours, showing that they are able to induce hypertrophy in rat neonatal cardiomyocytes. SCP showed a greater ability in increasing cell size than the other two peptides and was more

capable of increasing protein to DNA ratio, ANP and BNP mRNA levels than SRP. However, compared to UCN, SCP showed a significant difference only in its ability to increase mRNA levels of BNP. It was previously shown that the levels of ANP protein in rat neonatal cardiomyocytes, after UCN administration were higher than BNP, but the fold induction of BNP protein was higher than ANP (Ikeda et al., 1998).

As there was a slight difference in the effects on the mRNA levels of ANP and BNP between SCP and UCN it leads us to speculate that there must be a factor or factors downstream of the CRFR2 that has a higher effect on the BNP promoter. BNP promoter has been shown to be regulated differently than ANP promoter in the heart (Thuerauf and Glembotski, 1997). Where ANP promoter is highly inducible upon SAP and MEK kinases, BNP promoter appears to be more sensitive to Akt activation (Thureauf and Glembotski, 1997).

It could be that the differences observed in the levels of BNP and ANP mRNA upon treatment with the various UCN homologues is dependent on the activation of Akt and, that possibly, SCP is more effective in Akt activation than the other homologues. A closer examination and study of the two promoters might be able to shed some light on possible factors that are downstream of the Akt and activated after UCN homologues treatment to induce BNP/ANP expression. It would lead to results that might enable us to control the hypertrophic effects of the UCN peptides without compromising the cardioprotective effects.

To test this hypothesis and in order to understand the action of the UCN homologues in cardiomyocytes, we tried to characterise the differences in the activation of extracellular signal kinases and PI3 kinases upon stimulation by the UCN homologues. UCN was previously shown to be able to activate MAPK and Akt and that both pathways were important for cardiomyocyte survival after hypoxia/ reoxygenation injury. Additionally, the pathways that are involved in cardiomyocyte hypertrophy by UCN have not been examined. The only information available at the time the work was conducted was that the MAPK and p38 kinases are not involved in cardiomyocyte hypertrophy (Railson et al., 2002).

Although, previous studies on UCN hypertrophic effects speculated that PKA activation was important for these effects, this is the first report to show a distinct mechanism for the UCN induced hypertrophy. It is clear that PI3K and Akt activation in particular is at least one of the factors required not only for the hypertrophic effects of the UCN peptides (Fig. 5.4), but for cardioprotection as well. Akt activation is known to be an important factor in hypertrophy (Frey and Olson, 2003). Possible downstream effectors include such regulators of hypertrophy as GSK3β and mTOR. CRF has already been shown to regulate GSK3β expression in neuronal cells (Bayatti et al., 2003).

We previously speculated that Akt and MAPK pathways converge at a point downstream of the CRFR2. This notion is strengthened by the results presented

here. Akt phosphorylation (Fig. 5.1) was increased after blocking MAPK and in addition, in some of the experiments, blocking MAPK increased protein synthesis and cell area after peptide treatment (Fig. 5.3). These increases were not statistically significant however, apart from the effect of SCP on cell area (Fig. 5.3 Top and Bottom) and in the case of SRP when cells were transfected with DNMEK1 (Fig. 5.3 Bottom). These results indicate that there is a convergence of the two pathways upstream of MEK1 and that the MAPK pathway might act as a negative feedback loop of the Akt pathway stimulation on cell area after the peptide activation of the CRFR2, and probably only after an increased stimulation of Akt, as is the case with SCP and SRP. However, this hypothesis needs to be tested.

It has been previously shown that certain isoforms of the PI3K can be activated by Gsq trimeric proteins and also PI3K is able to induce MAPK activation either by interacting with ras, or by activating protein kinase C, which in consequence acts on raf-1 to activate the MAPK pathway (Bondeva et al., 1998; Graness et al., 1998; Clerk and Sugden 1999; Bayatti et al., 2003; Bayer et al, 2003; Foncea et al., 2000; Wetzker and Bohmer, 2003). In addition, protein kinase A, which is activated by Gs, can stimulate Akt independently of PI3K (Sable et al., 1997; Filippa et al., 1999).

In conclusion, we present a schematic diagram of the pathways that are stimulated by UCN and its homologues in the rat neonatal cardiomyocyte (Fig.

8.1). We believe that the UCN peptides after stimulation of the receptor lead to cAMP release and activation of PKA, MAPK and PI3K. Cyclic AMP can induce ras activation that can lead to activation of Akt either independently, or through PI3K. In addition ras activation can lead to MAPK activation either through PI3K and PKC or independently through raf-1. Two possible negative feedback loops are also presented. One negative feedback loop working through p38 on MAPK and another on Akt through MEK-1. This is the first time that the possible action of UCN and its homologues downstream of the receptor have been charted. It is by no means a definitive path, but it can be a basis for further exploration of the signalling events in the cardiomyocyte, following stimulation of the CRF receptor.

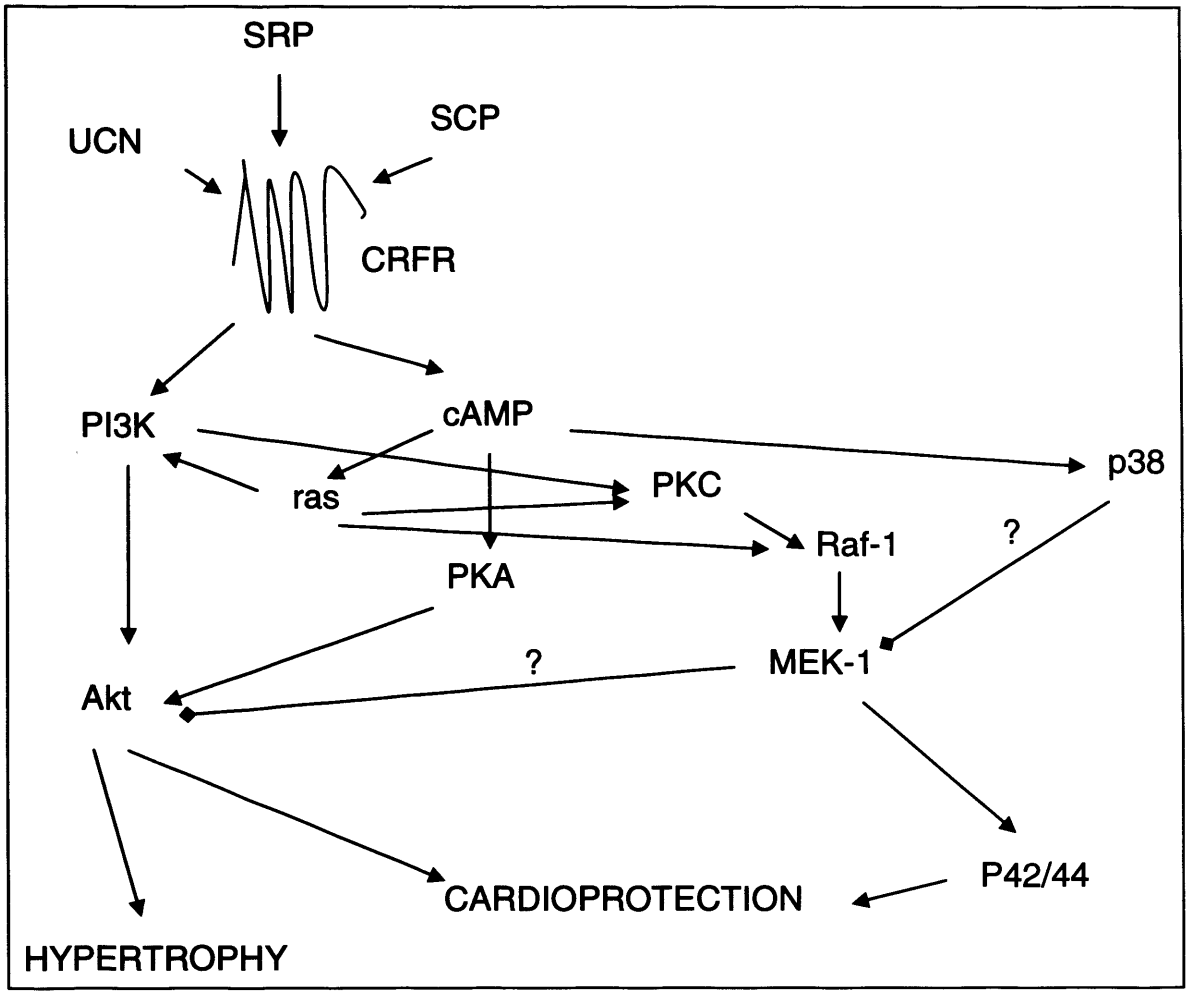


FIGURE 8.1. Schematic representation of the possible signalling events following activation of CRF receptor in rat neonatal cardiomyocytes by UCN homologues. UCN: urocortin, SCP: Stresscopin, SRP: stresscopin related peptide, PI3K: Phospho-inositol 3 kinase, cAMP: cyclic adenine monophosphate nucleotide, PKC: protein kinase C, PKA: Protein kinase A, CRFR: CRF receptor, arrows represent activation and clubs represent inhibition. Pathways that are not certain are presented with a questionmark.

Having shed light on the events that follow CRFR activation, we were interested in finding possible molecules that are downstream effectors of the physiological effects of the UCN homologues. For this reason a number of molecules, which were identified by an affymetrix gene chip assay, were tested for their involvement in the cardioprotective effects of the peptides. The molecules selected were PKC_{ε}, $iPLA_2$ and Kir 6.1. The three molecules above were selected because of their known implication in ischaemia reperfusion injury in the heart.

PKC is a protein kinase that has been previously shown to be involved in all aspects of cardiac physiology, including ischaemic preconditioning and hypertrophy. We have shown that PKC epsilon is one of the downstream targets of UCN and its homologues as its levels rise after peptide administration for 24 hours in rat neonatal cardiomyocytes. PKC_{ε} has been shown to be involved in cardioprotection after ischaemia reperfusion injury in neonatal (Chen et al. 1999; Dorn et al., 1999) and in adult cardiomyocytes (Dorn et al., 1999). Furthermore, PKC_{ε} has been shown to be involved in cardiac hypertrophy (Clerk et al., 1994). We are able to show that UCN and its homologues are able to induce the levels of PKC_{ε} without affecting the levels of the PKC_{δ} isoform in neonatal cardiomyocytes. In addition, UCN was able to induce rapid translocation of PKC-$_{\varepsilon}$, with no significant effect on the delta isoforms (Lawrence et al., 2005). PKC_{ε} translocation is a necessary step for MAPK p42/44 activation and could explain the necessity of MAPK in the cardioprotective effects of UCN.

Interestingly, as it was previously shown only the $iPLA_2$ isoform is involved in induction of apoptosis after oxidative stress (Martinez and Moreno, 2001). We are able to show that UCN and its homologues are able to attenuate the levels of $iPLA_2$ following hypoxia/reperfusion in rat neonatal cardiomyocytes. SCP was the most potent of the three peptides once more followed by UCN and SRP. We were also able to show that UCN homologues act in a similar manner to BEL an inhibitor of $iPLA_2$ as co-treatment of the homologues with the chemical inhibitor had no additional benefit to cell survival. Furthermore, UCN homologues were able to attenuate the amount of cell death caused by the addition of LPC, a product $iPLA_2$. Taken together these results suggest that UCN and its homologues act not only by reducing the amounts of $iPLA_2$ in the cell, but also by somehow inhibiting the release of the phospholipase metabolites arachidonic acid and LPC and blocking their deleterious effects. It is possible that UCN might be acting on other factors that are required for the mopping up of the phospholipase metabolites, or that the fact that it inhibits the positive feedback loop caused by LPC release in the induction of $iPLA_2$, is enough for the cellular mechanisms to cope with the harmful metabolites. This is certainly an area that requires further attention in the future.

The question that arises is this, are UCN effects on $iPLA_2$ only to inhibit the apoptotic effects of this molecule or is it somehow linked to lipid metabolism and maintenance of membrane integrity? Further work is certainly needed. In addition are the effects on $iPLA_2$ to minimise possible activation of other PKC isoforms? It

is not hard to speculate that phosphatidyl serine can be easily produced from lysopshosphatidyl serine. PS is one of the activators of PKC and the effects of UCN on $iPLA_2$ could be to control PKC activation and to minimise activation of other PKC isoforms than PKC epsilon.

.

We also examined the effects of UCN and its homologues on an ion channel the inward rectifying potassium subunit Kir 6.1. There are two types of K_{ATP} channels in the myocardium, sarcolemmal and mitochondrial. The sarcolemmal channel has been cloned and shown to be an octameric complex consisted of four pore forming Kir 6.2 subunits and four SUR2A sulfonyl urea receptors (Day et al., 1999; Gross and Fryer, 1999).

We have shown for the first time that the peptides are able to increase the levels of Kir6.1, and we provide evidence that this effect is specific for UCN as the sulfonyl urea subunit SUR and the isoform Kir 6.2 are not affected by UCN. We also show the importance of a functional Kir6.1 on the effects of UCN as transfection of a dominant negative form of Kir 6.1 blocked the protective effects of UCN. Moreover, for the first time we show that the mitochondrial K_{ATP} channel is important in myocardial viability after hypoxia reoxygenation and that the sarcolemmal channel is not affected by UCN or its homologues.

But why are UCN and its homologues leading to opening of K_{ATP} channels? It was previously shown that opening of mitochondrial K_{ATP} channels is one of the

steps during ischaemic preconditioning. K_{ATP} channel opening lead to a flux of K^+ through the mitochondrial membrane and accumulation of K^+ in the mitochondrion. The ion flux can lead to production of ROS that are mimicking ischaemic injury and preconditioning the cardiac cell for ischaemic episodes. But is K_{ATP} channel opening important for blocking apoptosis. Is the K^+ influx in the mitochondria necessary to keep mitochondrial integrity or is it a way for the cell to counteract the excess Ca^{+2} released in the sarcoplasm after an ischaemic episode? Do K_{ATP} channels open during ischaemia, or is it not a normal cell response to oxidative stress. We know that mitochondrial K_{ATP} channel opening is one of the ways that cardiomyocytes can become preconditioned, as was shown by diazoxide treatment, but we need to find out the role of the channel during ischaemia. Again further work is needed to clarify the role of K_{ATP} in cardioprotection.

This work is by no means complete. We have just started to realise the way the UCN peptides bring about their action in the cell. If we are to construct a cardioprotective agent based on the UCN peptides we need to find out if the hypertrophic effects examined are going to be crucial in stopping us using the peptides long-term as a protective drug for cardiac ischaemia. This needs testing in whole animals to determine the level of hypertrophy induced.

For this reason, we need to examine the effect of the peptides on the size of adult hearts. This could be done by intravenous administration of the peptides in whole animals and examining the effect of the peptides on the heart size on

regular time intervals and plot out the long term effects of UCN administration on heart size and more importantly on the ventricular and atrial wall muscle. Hearts would be removed for histochemical analysis and for RNA sampling to detect a change in the gene expression pattern of the heart after the UCN treatment. We will be looking for a possible upregulation of such hypertrophic gene markers as BNP and ANP.

It is not unthinkable that a peptide might be constructed that is not as potent in inducing PI-3K pathway. This is probably highly unlikely unless the activation of the PI3K pathway is dependent on certain stereochemical changes of the CRHR2 receptor that could possibly bring about a direct activation of PI3-K. It will be of great value in examining truncated SCP peptides to see the effects of the different parts of the molecule on the activation of the CRH receptor. It is an added benefit that SCP and SRP are exclusively binding to the CRFR2 and have no affinity to CRFR1. Apart from making possible studies easier as we only have to examine the effects on a single type receptor, SCP and SRP will have no effect on the brain via the CRFR1. This single characteristic of the SCP and SRP peptides makes them perfect candidates over UCN for the construction of synthetic peptides for therapeutic use.

For the last part, we examined the role of a novel gene called SAG in the neonatal cardiomyocytes that we show to be induced by UCN and its homologues. SAG is a 13kD RING finger protein that was shown to be

expressed in the heart among other tissues and it was specifically cloned as a response gene to oxidative stress. For this reason we believed that it would be a good candidate gene to examine in the context of myocardial ischaemia/reoxynation injury.

We were able to show that SAG mRNA and protein is expressed in the rat neonatal cardiomyocytes and that the levels of mRNA and protein are induced within an hour of ischaemia and are also present for the first hours of reoxygenation. Cardiomyocytes transfected with SAG are able to attenuate the amount of cell death after hypoxia/reoxygenation injury, and that the trasfection of a mutant SAG, with a mutation on the RING finger, failed to attenuate cell death, indicating the importance of the RING finger in the protective functions of SAG. Moreover, we were able to show that SAG is an important gene for cardiomyocytes, as transfection of an antisense SAG construct in the cells caused the cells to die.

SAG localisation changes after hypoxia/reoxygenation, and that a cytoplasmic SAG localisation is important for cell survival and that hypoxia triggers SAG exit from the nucleus. We propose that SAG is able to regulate the levels of caspase-3 in the cytoplasm of the cells and that this interaction is the reason for the importance of the cytoplasmic SAG in cell protection.

SAG is a very interesting molecule to examine more closely. The translocation of SAG from nucleus to the cytoplasm needs to be examined in more detail. There

three different possible mechanisms for SAG translocation. SAG might be able to shuttle in and out of the nucleus freely as it is of a small enough size. Adding a large epitope on the peptide might stop it from translocating to the cytoplasm. The lack of a possible nuclear –entry/ -exit signal, strengthens this hypothesis. SAG could also be able to exit the nucleus by attachment to another protein. This is the case with most proteins that make up the SCF E3 ligases for example cullin. SAG might be able to get in and out of the nucleus as part of the ligase. Lastly, the SAG translocation might be regulated by some kind of protein modification. SAG appears to contain a Casein kinase II phosphorylation signal. Kim et al., have shown that CKII phosphorylation of SAG is important for cell proliferation in HeLa cells and that SAG RING finger needs to be intact for the phosphorylation to take place. It would be of interest to examine the effect of CKII phosphorylation on SAG localisation and apoptosis.

The role of caspase-3 and SAG possible interaction needs to be established. If there is an interaction of SAG and caspase-3 then we would be able to show that by immunoprecipitation. The possibility of a regulation of a caspase by degradation is really exiting as it will show additional RING finger proteins involved in caspase regulation besides IAPs.

How can we explain the role of UCN in the cell? Why are there at least 3 different peptides that appear to do the same work in the cell and why is there such a multitude of signalling molecules activated by UCN and its homologues? Are all these signalling molecules interlinked and are all of them required for the actions

of UCN? These are the main questions that have come to the surface from this work and we will try to put forth a few explanations for them.

Nothing in a cell, and extrapolating to a tissue organ or organism, is maintained with no reason. Not after millions of years of evolutionary trials that have put all these mechanisms that are keeping the cell working in an ordinary fashion to the test. CRF peptides and among them UCN and its homologues appear to have similarities to the insect diuretic peptides. These peptides act on the Malpighian tubules of insect after feeding and induce H_2O removal and diuresis. So they have a role in homeostasis. Does that mean that CRF peptides have been evolved from a common ancestor between insects and vertebrates? That would indicate an evolutionary history of at least 450 million of years and that CRF peptides have evolved as osmoregulatory homeostatic mechanisms and their functions have evolved into a wider stress regulation response mechanism, that includes the fight or flight behavioural response that is manifested by the activation of the adrenal axis. We have already proposed that the presence of SCP and SRP could be evolutionary steps of having the stress coping mechanism activated on demand rather on maintaining low levels of the CRF/UCN peptides and controlling their function by the CRF binding peptide.

It would be interesting to find out if there is a binding peptide for SCP and SRP. Our theory would suggest that such a peptide could be absent, however it might

still be necessary in order to minimise the effects of secreted SCP and SRP as they do work in an autocrine/paracrine manner.

So, SCP and SRP could be evolutionary steps in dissecting the stress response to the adrenal axis as a behavioural response, and the SRP, SCP response to the periphery with mainly cellular stress coping mechanisms rather than whole body stress coping. It is of interest that UCN has been shown to have anxiolytic effects in contrast to the anxiogenic effects of the CRF. Another opposing effect of CRF and UCN is on depression, where CRF induces and UCN decreases depression (Rademaker, 2002). These differences are reflected upon the evolution and tissue distribution of the CRF receptors as well, where CRFR1 is localised in the brain and the CRFR2 is produced in the periphery.

During stress a cell has two option cope or die. It is easy to see then why UCN and each homologue have such antiapoptotic properties. UCN is there to give cells a chance to either maintain cellular function, or die if it can not cope anymore. It gives the chance to the cell to check if it has enough resources to see it through the stress period or if it is far gone to die. That also explains the fact why death is still observed in the presence of UCN.

UCN and its homologues are able to block apoptosis induced by ischaemia reperfusion in the heart. To do that we have shown that the activation of several signalling molecules are required to be activated. We have also shown that all

the signalling molecules that are activated by UCN peptides are required for UCN to be able to elicit its antiapoptotic effects. This indicates that all the signalling molecules are either downstream of a common activating molecule, such as PKA, and or they are communicating between them and that they are all required to activate all the necessary factors that are needed by UCN peptides to attenuate apoptosis. So, the cardioprotection elicited of UCN peptides is not the result of a single pathway, but of several pathways with probably PKA on the top of the signalling pyramid.

Interestingly some of the down-effector signalling molecules were recently shown by Lawrence to be located in the mitochondrion and to be important to maintain mitochondrial integrity (Lawrence et al., 2005). These molecules are PKC epsilon, Kir6.1 and $iPLA_2$. It is interesting to hypothesise that all these molecules are regulated by UCN peptides in order to maintain mitochondrial integrity in order to block the mitochondrial apoptosome to be formed and to guarantee mitochondrial functionality and production of ATP for cell viability. Also, we can hypothesise that $iPLA_2$ activity and levels are downregulated by UCN peptides not only to block the production of arachidonic acid and blocking apoptosis induced by $iPLA_2$ activity but also to maintain mitochondrial and cellular membrane integrity. The possible mechanism by which UCN and its homologues might act on the mitochondrion is shown on figure 8.2.

The effect of UCN peptides on Kir 6.1 could also have the additional effect in maintaining mitochondrial membrane potential and to control cytoplasmic osmotic pressure. That would link it back to the role it has in the insects. In conclusion, UCN peptides need to activate a number of signalling molecules in order to bring about their antiapoptotic and homeostatic effects.

Further work is needed to elucidate the theory present concerning the effects of UCN on the mitochondrion through PKC_{ε} translocation. We need to examine whether blocking PKC_{ε} translocation would have any effects on Kir 6.1 levels after UCN administration by western blotting. Through voltage gating experiments on isolated mitochondria we might be able to find out whether blocking PKC epsilon has any effect on the K^+ influx that is expected after UCN treatment. Experiments in $PKC_{\varepsilon}^{-/-}$ animals would show the effect of PKC epsilon on the Kir6.1 and K_{ATP} channel. Similarly, with the same experimental approach we can show the possible link between PKC epsilon and $iPLA_2$.

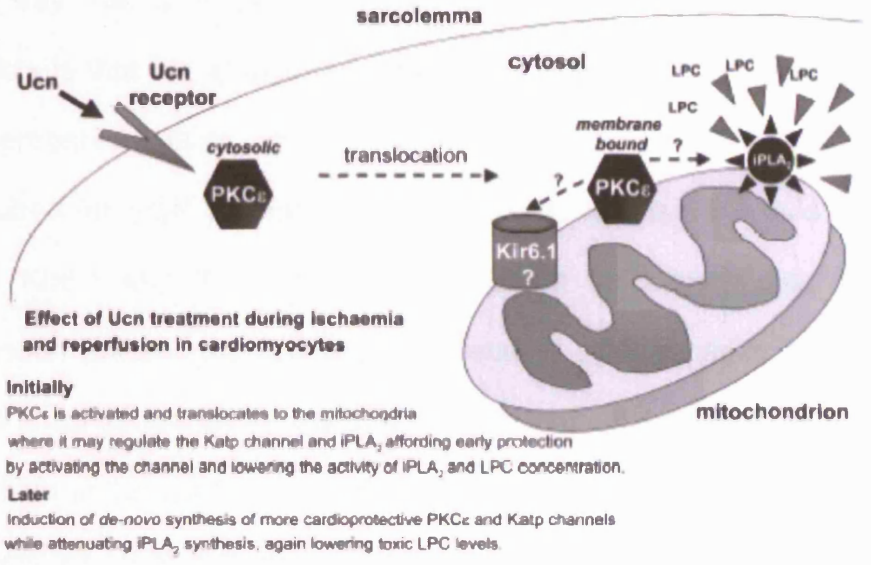


Figure 8.2. Effects of UCN on the mitochondrion. UCN treatment leads to PKC_{ε} translocation from the cytosol to the mitochondrion. There it might be able to regulate the K_{ATP} channel and lower the activity of $iPLA_2$ and thus maintaining mitochondrial membrane integrity and function. Longer term effects of UCN administration leads to additional PKC_{ε} synthesis, attenuation and lowering of $iPLA_2$ levels and increase in the levels of K_{ATP}, securing thus mitochondrial function.

UCN peptides are able to induce hypertrophy, as well. A big difference between the way that UCN peptides bring about their antiapoptotic and hypertrophic effects is that not all signalling molecules regulated by UCN are required for its hypertrophic effects. We are certain that MAPK p42/44 for example is not required for UCN peptide induced hypertrophy. Also, it would be hard to think that Kir6.1 and $iPLA_2$ might be important for hypertrophy. That raises the question whether the hypertrophic effects of UCN peptides are a necessary evil and a consequence of the activation of certain pathways, like Akt, that have intrinsic antiapoptotic and proliferative effects. Could it be that the hypertrophic effects of UCN peptides in the heart is a consequence of the inability of cardiomycytes to proliferate? It has been shown that UCN has proliferative effects in other cells (Ikeda et al., 2002). It could be that blocking of apoptosis is intrinsically linked with growth and proliferation, and you can not avoid blocking apoptosis without causing either cellular growth or proliferation. It could be an unavoidable risk a cell might have to take in order to survive the stress that caused UCN production. After all, stress should only last a brief period of time in the life of a cell and the main aim of the cell is to survive that period of stress the best it can until more favourable conditions are available.

As a final note we managed to compare the effects of the UCN homologous peptides in the rat neonatal cardiomyocyte and to provide an outlook of their action in the cell in regard to the antiapoptotic and hypertrophic effects of the

peptides. We also managed to show a mechanism of action for the three peptides and show some of possible targets of the peptides.

ACKNOWLEDGEMENTS

A PhD is an enormous task that it is not that dissimilar to a journey into the unknown, a great expedition of discovery, where even your destination might not be fully defined until you reach it. Along this journey there are many treacherous paths, twisty turns and dead ends and when you reach those points a helping hand, which will assist you and point you in the right direction, is always welcomed. So, this is the part where I express my enormous gratitude to all those people that have been an active part of my PhD journey.

First and foremost, my eternal love, respect and appreciation to my constant companion and compass in my life for the last twelve years, Rachel Davidge. Rachel, you have been a rock for me to shelter during my struggles along the road. You have been there for me to put me on my right path when I was straying, encouraged my achievements, been my lighthouse and kept a home for me to return to. Words cannot express your input on my endeavours. This PhD belongs to you as much as it does to me.

To my supervisors Prof DS Latchman and A Stephanou, for giving me the chance to do a PhD in the first place and for pointing out the errors of my ways and fuelling my positive instincts.

To Dr K. Lawrence, Dr TM Scarabelli and Prof R. Knight for their friendship and collaborations, to Dr M Callisano for his company and the pleasant and knowledgeable discourse on all matters biological.

To S. Irshad, YJ Patel, A Zourlidou and MM Muqit for their company during the late hours in the lab and for keeping me sane when things were going pear shaped.

To S. Davidson for all his help on all matters immunofluorescent and to T. Scarabelli for his assistance and demonstrations on whole heart experiments.

To Dr BK Brar for welcoming me in the lab and teaching me how to isolate rat neonatal primary cardiomyocytes.

Also, an enormous recognition to the British Heart Foundation for their funding. Whithout the help of such generous funding bodies, like BHF, research in the UK would not have been anywhere near the standard that it is today.

David and Kay Davidge thank you for your friendship and support. You have been my adopted family ever since I first came to England. I will forever be indebted to you.

Last as always, my acknowledgment for the contribution of my parents Vasilios and Efstatia Chanalaris. Thank you for always helping me without expecting anything in return. Σας αγαπώ πολύ!

By no means is this a complete list and apologies to all that I have forgot to mention here. Some of you have been acknowledged on my published papers and for the rest I can only say that I am deeply ashamed and you are invited to point my shortcoming next time we meet.

REFERENCES

Abdelrahman AM, Pang CC. 2003. Regional haemodynamic effects of Urocortin in the anaesthetized rat. *Eur. J. Pharmacol.* 466(3):317-21.

Adachi K, Lakka V, Zhao Y, Surrey S. 2004. Ubiquitylation of nascent globin chains in a cell-free system. *J Biol Chem.* 279 (40): 41767-74.

Albert CJ, Ford DA. 1999. Protein kinase C translocation and PKC-dependent protein phosphorylation during myocardial ischemia. *Am. J. Physiol* 276(2 Pt 2):H642-H650

Aoki H, Kang PM, Hampe J, Yoshimura K, Noma T, Matsuzaki M, Izumo S. 2002. Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. *J. Biol. Chem.* 277(12):10244-50

Ashraf M. 1997. Myocardial Ischaemia-Reperfusion injury: Free Radicals. In *Oxygen Radicals in the Disease Process*, ed. Thomas GE, Kalyanaraman B, 175-218 pp. Amsterdam; The Netherlands: Harwood Academic Publishers; The Gordon and Breach Publishing Group. 175-218 pp.

Atsumi G, Tajima M, Hadano A, Nakatani Y, Murakami M, Kudo I. 1998. Fas-induced arachidonic acid release is mediated by Ca2+-independent phospholipase A2 but not cytosolic phospholipase A2, which undergoes proteolytic inactivation. *J. Biol. Chem.* 273(22):13870-7

Baczko I, Giles WR, Light PE. 2004. Pharmacological activation of plasma-membrane KATP channels reduces reoxygenation-induced Ca(2+) overload in cardiac myocytes via modulation of the diastolic membrane potential. *Br. J. Pharmacol.* 141(6):1059-67.

Bailly V, Lauder S, Prakash S, Prakash L. 1997. Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjucating, DNA binding, and ATP hydrolytic activities. *J Biol Chem.* 272 (37): 23360-5.

Baines CP, Liu GS, Birincioglu M, Critz SD, Cohen MV, Downey JM. 1999. Ischemic preconditioning depends on interaction between mitochondrial KATP channels and actin cytoskeleton. *Am. J. Physiol* 276(4 Pt 2):H1361-H1368.

Baldi L, Brown K, Franzoso G, Siebenlist U. 1996. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκBα. *J Biol Chem.*271 (1): 376-79.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF. 2000. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat. Genet.* 24(4):410-4

Barr LF, Campbell SE, Baylin SB. 1997. Protein kinase C-beta 2 inhibits cycling and decreases c-myc-induced apoptosis in small cell lung cancer cells. *Cell Growth Differ.* 8(4):381-92.

Bayatti N, Zschocke J, Behl C. 2003. Brain region specific neuroprotective action and signalling of corticotropin-releasing hormone in primary neurons. *Endocrinology.* 144 (9): 4051-60.

Bayer AL, Heidkamp MC, Howes AL, Hellen Brown J, Byron KL, Samarel AM. 2003. Protein kinase C epsilon dependent activation of praline rich tyrosine kinase 2 in neonatal rat ventricular myocytes. *J Mol Cell Cardiol.* 35 (9): 1121-33.

Berra E, Municio MM, Sanz L, Frutos S, Diaz-Meco MT, Moscat J. 1997. Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. *Mol. Cell Biol.* 17(8):4346-54.

Besnard-Guerin C, Belaidouni N, Lassot I, Segeral E, Jobart A, Marchal C, Benarous R. 2004. HIV-1 Vpu sequesters beta-transducin repeat-

containing protein (betaTrCP) in the cytoplasm and provokes the accumulation of beta-catenin and other SCFbetaTrCP substrates. *J Biol Chem.* 279 (1): 788-95.

Bogoyevitch MA. 2000. Signalling via stress-activated mitogen-activated protein kinases in the cardiovascular system. *Cardiovasc. Res.* 45(4):826-42

Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP. 1998. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science.* 282 (5387): 293-6.

Brar BK, Stephanou A, Okosi A, Lawrence KM, Knight RA, Marber MS, Latchman DS. 1999. CRH-like peptides protect cardiac myocytes from lethal ischaemic injury. *Mol. Cell Endocrinol.* 158(1-2):55-63

Brar BK, Jonassen AK, Stephanou A, Santilli G, Railson J, Knight RA, Yellon DM, Latchman DS. 2000. Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. *J. Biol. Chem.* 275(12):8508-14

Brar BK, Railson J, Stephanou A, Knight RA, Latchman DS. 2002a. Urocortin increases the expression of heat shock protein 90 in rat cardiac myocytes in a MEK1/2-dependent manner. *J. Endocrinol.* 172(2):283-93

Brar BK, Stephanou A, Knight R, Latchman DS. 2002b. Activation of protein kinase B/Akt by urocortin is essential for its ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death. *J. Mol. Cell Cardiol.* 34(4):483-92

Brar BK, Jonassen AK, Egorina EM, Chen A, Negro A, Perrin MH, Mjos OD, Latchman DS, Lee KF, Vale W. 2004a. Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart. *Endocrinology* 145(1):24-35

Brar BK, Chen A, Perrin MH, Vale W. 2004b. Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides. *Endocrinology* 145(4):1718-29

Braz JC, Bueno OF, De Windt LJ, Molkentin JD. 2002. PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). *J. Cell Biol.* 156(5):905-19

Brown WJ, Chambers K, Doody A. 2003. Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. *Traffic.* 4(4):214-21

Chen A, Wu K, Fuchs SY, Tan P, Gomez C, Pan ZQ. 2000. The conserved RING-H2 finger of ROC1 is required for ubiquitin ligation. *J. Biol. Chem.* 275(20):15432-9

Chen CH, Gray MO, Mochly-Rosen D. 1999 Cardioprotection from ischemia by a brief exposure to physiological levels of ethanol: role of epsilon protein kinase C. *Proc Natl Acad Sci U S A.* 96(22):12784-9.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW, Mochly-Rosen D. 2001. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc. Natl. Acad. Sci. U. S. A* 98(20):11114-9

Cheng W, Kajstura J, Nitahara JA, Li B, Reiss K, Liu Y, Clark WA, Krajewski S, Reed JC, Olivetti G, Anversa P. 1996. Programmed myocyte cell death affects the viable myocardium after infarction in rats. *Exp. Cell Res.* 226(2):316-27

Clerk A, Bogoyevitch MA, Anderson MB, Sugden PH. 1994. Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. *J. Biol. Chem.* 269(52):32848-57

Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, Quon MJ. 1997. Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol. Endocrinol.* 11(13):1881-90

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P, Stenzel-Poore MP. 2000. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat. Genet.* 24(4):403-9

Cui Y, Giblin JP, Clapp LH, Tinker A. 2001. A mechanism for ATP-sensitive potassium channel diversity: Functional coassembly of two pore-forming subunits. *Proc. Natl. Acad. Sci. U. S. A* 98(2):729-34.

Day YJ, Gao Z, Tan PC, Linden J. 1999. ATP-sensitive potassium channel and preconditioning. *Acta Anaesth. Sin.* 37:121-131.

Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, Messing RO. 2000. Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol Lung Cell Mol. Physiol* 279(3):L429-L438.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugatingenzyme complex and a unique polyubiquitin chain. *Cell.* 103 (2): 351-61.

Di Fiore PP, Polo S, Hofmann K. 2003. When ubiquitin meets ubiquitin receptors: a signalling connection. *Nature Rev Mol Cell Biol.* 497 (4): 491-7.

Disatnik MH, Buraggi G, Mochly-Rosen D. 1994. Localization of protein kinase C isozymes in cardiac myocytes. *Exp. Cell Res.* 210(2):287-97

Donjerkovic D, Mueller CM, Scott DW. 2000. Steroid- and retinoid-mediated growth arrest and apoptosis in WEHI-231 cells: role of NF-kappaB, c-Myc and CKI p27(Kip1). *Eur. J. Immunol.* 30(4):1154-61

Dorn GW, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, Mochly-Rosen D. 1999. Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. *Proc. Natl. Acad. Sci. U. S. A* 96(22):12798-803.

Duan H, Wang Y, Aviram M, Swaroop M, Loo JA, Bian J, Tian Y, Mueller T, Bisgaier CL, Sun Y. 1999. SAG, a novel zinc RING finger protein that

protects cells from apoptosis induced by redox agents. *Mol. Cell Biol.* 19(4):3145-55

Duan H, Tsvetkov LM, Liu Y, Song Y, Swaroop M, Wen R, Kung HF, Zhang H, Sun Y. 2001. Promotion of S-phase entry and cell growth under serum starvation by SAG/ROC2/Rbx2/Hrt2, an E3 ubiquitin ligase component: association with inhibition of p27 accumulation. *Mol. Carcinog.* 30(1):37-46

Emoto Y, Kisaki H, Manome Y, Kharbanda S, Kufe D. 1996. Activation of protein kinase Cdelta in human myeloid leukemia cells treated with 1-beta-D-arabinofuranosylcytosine. *Blood* 87(5):1990-6

Engelmann GL, Birchenall-Roberts MC, Ruscetti FW, Samarel AM. 1993. Formation of fetal rat cardiac cell clones by retroviral transformation: retention of select myocyte characteristics. *J. Mol. Cell Cardiol.* 25(2):197-213.

Erhardt P, Schremser EJ, Cooper GM. 1999. B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. *Mol Cell Biol* 19: 5308-5315.

Finley D, Bartel B, Varshavsky A. 1989. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338(6214):394-401

Fliss H, Gattinger D. 1996. Apoptosis in ischemic and reperfused rat myocardium. *Circ. Res.* 79(5):949-56

Foncea R, Galvez A, Perez V, Morales MP, Calixto A, Melendez J, Gonzalez-Jara F, Diaz-Araya G, Sapag-Hagar M, Sugden PH, LeRoith D, Lavandero S. 2000. Extracellular regulated kinase, but not protein kinase C, is an antiapoptotic signal of insulin-like growth factor-1 on cultured cardiac myocytes. *Biochem Biophys Res Commun.* 273 (2): 736-44.

Freemont PS, Hanson IM, Trowsdale J. 1991. A novel cysteine-rich sequence motif. *Cell* 64(3):483-4

Frey N, Olson EN. 2003. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu. Rev. Physiol* 65:45-79.

Fuchs SY, Spiegelman VS, Kumar KG. 2004. The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. *Oncogene* 23 (11): 2028-36.

Fujii T, Garcia-Bermejo ML, Bernabo JL, Caamano J, Ohba M, Kuroki T, Li L, Yuspa SH, Kazanietz MG. 2000. Involvement of protein kinase C delta (PKCdelta) in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCdelta. *J. Biol. Chem.* 275(11):7574-82

Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ. 1997. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ. Res.* 81(6):1072-82

Gill C, Mestril R, Samali A. 2002. Losing heart: the role of apoptosis in heart disease--a novel therapeutic target? *FASEB J.* 16(2):135-46

Goldberg M, Zhang HL, Steinberg SF. 1997. Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. *J. Clin. Invest* 99(1):55-61

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest* 94(4):1621-8

Goyal L. 2001. Cell death inhibition: keeping caspases in check. *Cell* 104(6):805-8

Grammatopoulos DK, Randeva HS, Levine MA, Katsanou ES, Hillhouse EW. 2000. Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated via R1alpha and R2beta CRH receptor subtypes and stimulation of Gq-proteins. *Mol. Endocrinol.* 14(12):2076-91

Graness A, Adomeit A, Heinze R, Wetzker R, Liebmann C. 1998. A novel mitogenic signaling pathway of bradykinin in the human colon carcinoma cell line SW-480 involves sequential activation of a Gq/11 protein, phosphatidylinositol 3-kinase beta, and protein kinase Cepsilon. *J Biol Chem.* 273(48):32016-22.

Gray MO, Karliner JS, Mochly-Rosen D. 1997. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J. Biol. Chem.* 272(49):30945-51

Grenfell SJ, Trausch-Azar JS, Handley-Gearhart PM, Ciechanover A, Schwartz AL. 1994. Nuclear localization of the ubiquitin-activating enzyme, E1, is cell-cycle-dependent. *Biochem. J.* 300 (Pt 3):701-8.

Gross GJ, Fryer RM. 1999. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ Res.* 84: 973-999.

Grubel PA, Kolodgie F, Serebruany V, Mergner WJ. 1998. Myocardial Cell Injury During Ischaemia and Reflow. In *Principles of Medical Biology*, ed. Bittar EE, Bittar N,127-166 pp. Stamford;Connecticut;USA: JAI Press INC. 127-166 pp.

Grunt M, Haug C, Duntas L, Pauschinger P, Maier V, Pfeiffer EF. 1992. Dilatory and inotropic effects of corticotropin-releasing factor (CRF) on the isolated heart. Effects on atrial natriuretic peptide (ANP) release. *Horm Metab Res* 24 (2): 56-9.

Gusterson RJ, Jazrawi E, Adcock IM, Latchman DS. 2003. The transcriptional co-activators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. *J Biol Chem.* 278(9):6838-47.

Gusterson R, Brar B, Faulkes D, Giordano A, Chrivia J, Latchman D. 2002. The transcriptional co-activators CBP and p300 are activated via phenylephrine through the p42/p44 MAPK cascade. *J Biol Chem* 277 (4): 2517-24.

Handley-Gearhart PM, Stephen AG, Trausch-Azar JS, Ciechanover A, Schwartz AL. 1994. Human ubiquitin-activating enzyme, E1. Indication of potential nuclear and cytoplasmic subpopulations using epitope-tagged cDNA constructs. *J. Biol. Chem.* 269(52):33171-8

Haq S, Kilter H, Michael A, Tao J, O'Leary E, Sun XM, Walters B, Bhattacharya K, Chen X, Cui L, Andreucci M, Rosenzweig A, Guerrero JL, Patten R, Liao R, Molkentin J, Picard M, Bonventre JV, Force T. 2003. Deletion of cytosolic phospholipase A2 promotes striated muscle growth. *Nature Med.* 9(7):944-51

Haug C, Grunt M, Schmid S, Steinbach G, Metzele A, Maier V, Keck FS, Grunert, A. 1994 Effect of corticotropin releasing factor on atrial natriuretic peptide release from the isolated perfused rat heart. *Arzneimittelforschung* 44 (5): 579-82.

Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg, FM. 2003. International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol Rev* 55 (1): 21-6.

Heidkamp MC, Bayer AL, Martin JL, Samarel AM. 2001. Differential activation of mitogen-activated protein kinase cascades and apoptosis by

protein kinase C epsilon and delta in neonatal rat ventricular myocytes. *Circ Res.* 89(10):882-90.

Hicke L. 2001. Protein regulation by monoubiquitin. *Nature Rev Mol Cell Biol.* 2(3):195-201.

Hochstrasser M. 1996. Ubiquitin-dependent protein degradation. *Annu Rev Genet.* 30: 405-439.

Hofmann RM and Pickart CM. 1999. Noncanonical MMS2-encoded ubiquitin-conjucating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell.* 96 (5): 645-53.

Holmuhamedov EL, Wang L, Terzic A. 1999. ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria. *J. Physiol* 519 Pt 2:347-60.

Hoppe T, Matuschewski K, Rape M, Schlenker S, Ulrich HD, Jentsch S. 2000. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell.* 102 (5): 577-86.

Hreniuk D, Garay M, Gaarde W, Monia BP, McKay RA, Cioffi CL. 2001. Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2,

suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes. *Mol Pharmacol.* 59(4):867-74

Hsu SY, Hsueh AJ. 2001. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nature Med.* 7(5):605-11.

Hsueh CT, Wu YC, Schwatrz GK. 2001. UCN-01 suppresses E2F-1 mediated by ubiquitin-proteasome-dependent degradation. *Clin Cancer Res.* 7 (3): 669-74.

Huang Y, Duan H, Sun Y. 2001. Elevated expression of SAG/ROC2/Rbx2/Hrt2 in human colon carcinomas: SAG does not induce neoplastic transformation, but antisense SAG transfection inhibits tumor cell growth. *Mol Carcinog.* 30(1):62-70

Huh J, Gross GJ, Nagase H, Liang BT. 2001. Protection of cardiac myocytes via delta(1)-opioid receptors, protein kinase C, and mitochondrial K(ATP) channels. *Am J Physiol Heart Circ Physiol* 280(1):H377-H383.

Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci USA.* 92 (7): 2563-7.

Ikeda K, Tojo K, Sato S, Ebisawa T, Tokudome G, Hosoya T, Harada M, Nakagawa O, Nakao K. 1998. Urocortin, a newly identified corticotropin-releasing factor-related mammalian peptide, stimulates atrial natriuretic peptide and brain natriuretic peptide secretions from neonatal rat cardiomyocytes. *Biochem Biophys Res Commun.* 250(2):298-304

Ikeda K, Tojo K, Oki Y, Nakao K. 2002. Urocortin has cell-proliferative effects on cardiac non-myocytes. *Life Sci.* 71(16):1929-38.

Iwai K, Yamanaka K, Kamura T, Minato N, Conaway RC, Conaway JW, Klausner RD, Pause A. 1999. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc Natl Acad Sci U. S. A* 96(22):12436-41.

Jaattela M, Benedict M, Tewari M, Shayman JA, Dixit VM. 1995. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene* 10(12):2297-305

Jentsch S, Pyrowolakis G. 2000. Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol.* 10(8):335-42.

Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, Debatin KM. 2000. Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. *Circulation* 102(8):915-20

Jiao JH, Baumann P, Baron A, Roatti A, Pence RA, Baertschi AJ. 2000. Sulfonylurea receptor ligands modulate stretch-induced ANF secretion in rat atrial myocyte culture. *Am. J. Physiol Heart Circ. Physiol* 278(6):H2028-H2038.

Joazeiro CA, Weissman AM. 2000. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102(5):549-52.

Johnson GL, Lapadat R. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298(5600):1911-2.

Kagawa T, Varticovski L, Sai Y, Arias IM. 2002. Mechanism by which cAMP activates PI3-kinase and increases bile acid secretion in WIF-B9 cells. *Am J Physiol Cell Physiol* 283:C1655-C1666.

Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P. 1996. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 74(1):86-107.

Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P. 1998. Myocyte proliferation in end-stage cardiac failure in humans. *Proc. Natl. Acad. Sci. U. S. A* 95(15):8801-5.

Kaiser P, Flick K, Wittenberg C, Reed SI. 2000. Regulation of transcription by ubiquitylation without proteolysis: Cdc34/SCF^{Met30}-mediated inactivation of the transcription factor Met4. *Cell.*102 (3): 303-14.

Kamura T, Sato S, Haque D, Liu L, Kaelin WG, Jr., Conaway RC, Conaway JW. 1998. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev.* 12(24):3872-81.

Kamura T, Sato S, Iwai K, Czyzyk-Krzeska M, Conaway RC, Conaway JW. 2000. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc. Natl. Acad. Sci. U. S. A* 97(19):10430-5.

Kay B, Williamson MP, Sudol M. 2000. The importance of being proline: the interaction of proline-rich motifs in signalling proteins with their cognate domains. *FASEB J.* 14 (2): 231-41.

Keating MT, Sanguinetti MC. 2001. Molecular and cellular mechanisms of cardiac arrhythmias. *Cell* 104(4):569-80.

Kentsis A, Dwyer EC, Perez JM, Sharma M, Chen A, Pan ZQ, Borden KL. 2001. The RING domains of the promyelocytic leukaemia protein PML and the arenaviral protein Z repress translationby directly inhibiting translation initiation factor eIF4E. *J Mol Biol.* 312 (4): 609-23.

Kevelaitis E, Peynet J, Mouas C, Launay JM, Menasche P. 1999. Opening of potassium channels: the common cardioprotective link between preconditioning and natural hibernation? *Circulation* 99(23):3079-85.

Kihara N, Fujimura M, Yamamoto I, Itoh E, Inui A, Fujimiya M. 2001. Effects of central and peripheral urocortin on fed and fasted gastroduodenal motor activity in conscious rats. *Am J Physiol Gastrointest Liver Physiol* 280(3):G406-G419.

Kile BT, Alexander WS. 2001. The suppressors of cytokine signalling (SOCS). *Cell Mol. Life Sci.* 58(11):1627-35.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J. 2000. Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. *Nature Genet.* 24(4):415-9.

Kloetzel PM. 2001. Antigen processing by the proteasome. *Nature Rev Mol Cell Biol.* 2 (3): 179-87.

Kohno M, Kawahito Y, Tsubouchi Y, Hashiramoto A, Yamada R, Inoue KI, Kusaka Y, Kubo T, Elenkov IJ, Chrousos GP, Kondo M, Sano H. 2001. Urocortin expression in synovium of patients with rheumatoid arthritis and osteoarthritis: relation to inflammatory activity. *J Clin Endocrinol Metab.* 86(9):4344-52.

Kostich WA, Chen A, Sperle K, Largent BL. 1998. Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor. *Mol Endocrinol.* 12(8):1077-85.

Kotani K, Ogawa W, Hino Y, Kitamura T, Ueno H, Sano W, Sutherland C, Granner DK, Kasuga M. 1999. Dominant negative forms of Akt (protein kinase B) and atypical protein kinase Clambda do not prevent insulin inhibition of phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem.* 274(30):21305-12.

Krijnen PA, Nijmeijer R, Meijer CJ, Visser CA, Hack CE, Niessen HW. 2002. Apoptosis in myocardial ischaemia and infarction. *J Clin Pathol.* 55(11):801-11.

Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL, Glembotski CC, Quintana PJ, Sabbadini RA. 1996. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the

sphingolipid signaling cascade in cardiac cell death. *J Clin Invest.* 98(12):2854-65

Kulik G, Klippel A, Weber MJ. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol.* 17(3):1595-606.

Kuras L, Rouillon A, Lee T, Barbey R, Tyers M, Thomas D. 2002. Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. *Mol Cell.* 10(1):69-80.

Latchman DS. 2001. Urocortin protects against ischemic injury via a MAPK-dependent pathway. *Trends Cardiovasc Med.* 11(5):167-9.

Latchman DS. 2002. Urocortin. *Int J Biochem Cell Biol.* 34(8):907-10.

Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem.* 271(34):20608-16.

Lawrence KM, Kabir AM, Bellahcene M, Davidson S, Cao XB, McCormick J, Mesquita RA, Carroll CJ, Chanalaris A, Townsend PA, Hubank M, Stephanou A, Knight RA, Marber MS, Latchman DS. 2005.

Cardioprotection mediated by urocortin is dependent on PKCepsilon activation. *FASEB J.* 19 (7):831-3.

Lawrence KM, Chanalaris A, Scarabelli T, Hubank M, Pasini E, Townsend PA, Comini L, Ferarri R, Tinker A, Stephanou A, Knight RA, Latchman DS. 2002. K(ATP) channel gene expression is induced by urocortin and mediatesits cardioprotective effect. *Circulation.* 106 (12): 1556-1562.

Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW. 2001. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U. S. A* 98(13):7570-5.

Li J, Yang XK, Yu XX, Ge ML, Wang WL, Zhang J, Hou YD. 2000. Overexpression of p27(KIP1) induced cell cycle arrest in G(1) phase and subsequent apoptosis in HCC-9204 cell line. *World J Gastroenterol.* 6(4):513-21.

Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. 2003. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* 302(5652):1972-5.

Liao W, Yeung SC, Chan L. 1998. Proteasome-mediated degradation of apolipoprotein B targets both nascent peptides cotranslationally before translocation and full-length apolipoprotein B after translocation into the endoplasmic reticulum. *J Biol Chem.* 273 (42): 27225-30.

Lisztwan J, Imbert G, Wirbelauer C, Gstaiger M, Krek W. 1999. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes Dev.* 13(14):1822-33.

Liu H, Zhang HY, Zhu X, Shao Z, Yao Z. 2002. Preconditioning blocks cardiocyte apoptosis: role of K(ATP) channels and PKC-epsilon. *Am J Physiol Heart Circ Physiol.* 282(4):H1380-H1386.

Liu Y, Sato T, O'Rourke B, Marban E. 1998. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* 97(24):2463-9.

Loeb KR, Haas AL. 1994. Conjugates of ubiquitin cross-reactive protein distribute in a cytoskeletal pattern. *Mol Cell Biol.* 14(12):8408-19.

Lovejoy DA, Balment RJ. 1999. Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen Comp Endocrinol.* 115(1):1-22.

Lovering R, Hanson IM, Borden KL, Martin S, O'Reilly NJ, Evan GI, Rahman D, Pappin DJ, Trowsdale J, Freemont PS. 1993. Identification and preliminary characterization of a protein motif related to the zinc finger. *Proc Natl Acad Sci U. S. A* 90(6):2112-6.

Lucas M, Sanchez-Margalet V. 1995. Protein kinase C involvement in apoptosis. *Gen Pharmacol* 26(5):881-7.

Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue TL. 1999. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation* 99(13):1685-91.

Mackay K, Mochly-Rosen D. 1999. An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. *J Biol Chem.* 274(10):6272-9.

Mackay K, Mochly-Rosen D. 2000. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischemia. *J Mol Cell Cardiol.* 32(8):1585-8.

Mackay KB, Stiefel TH, Ling N, Foster AC. 2003. Effects of a selective agonist and antagonist of CRF2 receptors on cardiovascular function in the rat. *Eur J Pharmacol.* 469(1-3):111-5.

Mancuso DJ, Abendschein DR, Jenkins CM, Han X, Saffitz JE, Schuessler RB, Gross RW. 2003. Cardiac ischemia activates calcium-independent phospholipase A2beta, precipitating ventricular tachyarrhythmias in transgenic mice: rescue of the lethal electrophysiologic phenotype by mechanism-based inhibition. *J Biol Chem.* 278(25):22231-6.

Martinez J, Moreno JJ. 2001. Role of Ca2+-independent phospholipase A2 on arachidonic acid release induced by reactive oxygen species. *Arch Biochem Biophys.* 392(2):257-62.

Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, Force TL, Franke TF, Hajjar RJ, Rosenzweig A. 2001. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 104(3):330-5.

McCully JD, Levitsky S. 2003. The mitochondrial K(ATP) channel and cardioprotection. *Ann Thorac Surg.* 75(2):S667-S673.

Michel MC, Li Y, Heusch G. 2001. Mitogen-activated protein kinases in the heart. *Naunyn Schmiedebergs Arch Pharmacol.* 363(3):245-66.

Miura T, Liu Y, Kita H, Ogawa T, Shimamoto K. 2000. Roles of mitochondrial ATP-sensitive K channels and PKC in anti-infarct tolerance afforded by adenosine A1 receptor activation. *J Am Coll Cardiol.* 35(1):238-45.

Mizuno Y, Hattori N, Mori H, Suzuki T, Tanaka K. 2001. Parkin and Parkinson's disease. *Cur Opin Neurol.* 14 (4): 477-82.

Mori M, Uchida M, Watanabe T, Kirito K, Hatake K, Ozawa K, Komatsu N. 2003. Activation of extracellular signal-regulated kinases ERK1 and ERK2 induces Bcl-xL up-regulation via inhibition of caspase activities in erythropoietin signaling. *J Cell Physiol* 195: 290-297.

Murray NR, Fields AP. 1997. Atypical protein kinase C iota protects human leukemia cells against drug-induced apoptosis. *J Biol Chem.* 272(44):27521-4.

Murry CE, Jennings RB, Reimer KA. 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74(5):1124-36.

Nakatsuji Y, Miller RH. 2001. Selective cell-cycle arrest and induction of apoptosis in proliferating neural cells by ganglioside GM3. *Exp Neurol.* 168(2):290-9.

Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Khaw BA. 1996. Apoptosis in myocytes in end-stage heart failure. *N Engl J Med.* 335(16):1182-9.

Nishikimi T, Miyata A, Horio T, Yoshihara F, Nagaya N, Takishita S, Yutani C, Matsuo H, Matsuoka H, Kangawa K. 2000. Urocortin, a member of the corticotropin-releasing factor family, in normal and diseased heart. *Am J Physiol Heart Circ Physiol.* 279(6):H3031-H3039.

O'Rourke B. 2000. Pathophysiological and protective roles of mitochondrial ion channels. *J Physiol.* 529 Pt 1:23-36.

Oh H, Fujio Y, Kunisada K, Hirota H, Matsui H, Kishimoto T, Yamauchi-Takihara K. 1998. Activation of phosphatidylinositol 3-kinase through glycoprotein 130 induces protein kinase B and p70 S6 kinase phosphorylation in cardiac myocytes. *J Biol Chem.* 273(16):9703-10.

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG. 2000. Ubiquitination of hypoxia-inducible factor

requires direct binding to the β-domain of the von Hippel-Lindau protein. *Nature Cell Biol.* 2 (7): 423-7.

Ohsumi Y. 2001. Molecular dissection of autophagy: two ubiquitin-like systems. *Nature Rev Mol Cell Biol.* 2(3):211-6.

Ohta T, Michel JJ, Xiong Y. 1999. Association with cullin partners protects ROC proteins from proteasome-dependent degradation. *Oncogene* 18(48):6758-66.

Ohta T, Michel JJ, Schottelius AJ, Xiong Y. 1999. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol Cell* 3(4):535-41.

Okamoto S, Kimura K, Saito M. 2001. Anorectic effect of leptin is mediated by hypothalamic corticotropin-releasing hormone, but not by urocortin, in rats. *Neurosci Lett.* 307(3):179-82.

Okosi A, Brar BK, Chan M, D'Souza L, Smith E, Stephanou A, Latchman DS, Chowdrey HS, Knight RA. 1998. Expression and protective effects of urocortin in cardiac myocytes. *Neuropeptides* 32(2):167-71.

Oldenburg O, Qin Q, Krieg T, Yang XM, Philipp S, Critz SD, Cohen MV, Downey JM. 2004. Bradykinin induces mitochondrial ROS generation via

NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. *Am J Physiol Heart Circ Physiol.* 286(1):H468-H476.

Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. 2000. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res.* 87(6):460-6.

Pandey P, Avraham S, Kumar S, Nakazawa A, Place A, Ghanem L, Rana A, Kumar V, Majumder PK, Avraham H, Davis RJ, Kharbanda S. 1999. Activation of p38 mitogen-activated protein kinase by PYK2/related adhesion focal tyrosine kinase-dependent mechanism. *J Biol Chem.* 274(15):10140-4.

Parkes DG, Vaughan J, Rivier J, Vale W, May CN. 1997. Cardiac inotropic actions of urocortin in conscious sheep. *Am J Physiol.* 272 (5 Pt 2):H2115-H2122.

Parkes DG, May CN. 2000. Urocortin: A Novel Player in Cardiac Control. *News Physiol Sci.* 15:264-8.

Parkes DG, Weisinger RS, May CN. 2001. Cardiovascular actions of CRH and urocortin: an update. *Peptides* 22(5):821-7

Perrin MH, Vale WW. 1999. Corticotropin releasing factor receptors and their ligand family. *Ann NY Acad Sci.* 885:312-28.

Petersen S, Peto V, Rayner M. Coronary Heart Disease Statistics. British Heart Foundation Health Promotion Group and Department of Public Health. 2003 Edition. 2003. Oxford; UK, University of Oxford.

Pickart CM. 2000. Ubiquitin in chains. *Trends Biochem Sci.* 25 (11): 544-8.

Plant PJ, Lafont F, Lecat S, Verkade P, Simons K, Rotin D. 2000. Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIb. *J Cell Biol.* 149 (7): 1473-84.

Potuschak T, Stary S, Schlogelhofer P, Becker F, Nejinskaia V, Bachmair A. 1998. PRT1 of Arabidopsis thaliana encodes a component of the plant N-end rule pathway. *Proc Natl Acad Sci U. S. A.* 95(14):7904-8.

Prasad MR, Popescu LM, Moraru II, Liu XK, Maity S, Engelman RM, Das DK. 1991. Role of phospholipases A2 and C in myocardial ischemic reperfusion injury. *Am J Physiol.* 260(3 Pt 2):H877-H883.

Rademaker MT, Charles CJ, Espiner EA, Fisher S, Frampton CM, Kirkpatrick CM, Lainchbury JG, Nicholls MG, Richards AM, Vale WW. 2002. Beneficial hemodynamic, endocrine, and renal effects of urocortin in

experimental heart failure: comparison with normal sheep. *J Am Coll Cardiol.* 40(8):1495-505.

Radulovic M, Spiess J. 2001. Immunomodulatory role of the corticotropin-releasing factor. *Arch Immunol Ther Exp. (Warsz.).* 49(1):33-8.

Railson JE, Liao Z, Brar BK, Buddle JC, Pennica D, Stephanou A, Latchman DS. 2002. Cardiotrophin-1 and urocortin cause protection by the same pathway and hypertrophy via distinct pathways in cardiac myocytes. *Cytokine* 17(5):243-53.

Regan CP, Li W, Boucher DM, Spatz S, Su MS, Kuida K. 2002. Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. *Proc Natl Acad Sci U. S. A.* 99(14):9248-53.

Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE. 2001. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U. S. A.* 98(5):2843-8.

Reyland ME, Anderson SM, Matassa AA, Barzen KA, Quissell DO. 1999. Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem.* 274(27):19115-23.

Rubio I, Rodriguez-Viciana P, Downward J, Wetzker R. 1997. Interaction of Ras with phosphoinositide 3-kinase γ. Biochem J; 326:891-895.

Sable CL, Filippa N, Hemmings B, Van Obberghen E. 1997. cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. *FEBS Let* 109: 253-257.

Saraste A, Pulkki K. 2000. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res.* 45(3):528-37.

Sargent CA, Wilde MW, Dzwonczyk S, Tuttle JG, Murray HN, Atwal K, Grover GJ. 1996. Glyburide-reversible cardioprotective effects of calcium-independent phospholipase A2 inhibition in ischemic rat hearts. *Cardiovasc Res.* 31(2):270-7.

Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. 2000. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. *Circulation* 101(4):439-45.

Sasaki H, Yukiue H, Kobayashi Y, Moriyama S, Nakashima Y, Kaji M, Fukai I, Kiriyama M, Yamakawa Y, Fujii Y. 2001. Expression of the

sensitive to apoptosis gene, SAG, as a prognostic marker in nonsmall cell lung cancer. *Int J Cancer* 95(6):375-7.

Sato T, O'Rourke B, Marban E. 1998. Modulation of mitochondrial ATP-dependent K+ channels by protein kinase C. *Circ Res.* 83(1):110-4.

Sato T, Sasaki N, O'Rourke B, Marban E. 2000. Adenosine primes the opening of mitochondrial ATP-sensitive potassium channels: a key step in ischemic preconditioning? *Circulation* 102(7):800-5.

Scarabelli TM, Knight RA, Rayment NB, Cooper TJ, Stephanou A, Brar BK, Lawrence KM, Santilli G, Latchman DS, Baxter GF, Yellon DM. 1999. Quantitative assessment of cardiac myocyte apoptosis in tissue sections using the fluorescence-based tunel technique enhanced with counterstains. *J Immunol Methods* 228(1-2):23-8.

Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, Latchman DS. 2002a. Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. *Circ Res.* 90(6):745-8.

Scarabelli TM, Pasini E, Stephanou A, Comini L, Curello S, Raddino R, Ferrari R, Knight R, Latchman DS. 2002b. Urocortin promotes hemodynamic and bioenergetic recovery and improves cell survival in the

isolated rat heart exposed to ischemia/reperfusion. *J Am Coll Cardiol.* 40(1):155-61.

Scheffner M, Huibregtse JM, Viersta RD, Howley PM. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell.* 75 (3): 495-505.

Schulman D, Latchman DS, Yellon DM. 2002. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol.* 283(4):H1481-H1488.

Schwertz DW, Halverson J. 1992. Changes in phosphoinositide-specific phospholipase C and phospholipase A2 activity in ischemic and reperfused rat heart. *Basic Res Cardiol.* 87(2):113-27.

Shade RE, Blair-West JR, Carey KD, Madden LJ, Weisinger RS, Rivier JE, Vale WW, Denton DA. 2002. Ingestive responses to administration of stress hormones in baboons. *Am J Physiol Regul Integr Comp Physiol.* 282(1):R10-R18.

Sharma G-D, He J, Bazan EPH. 2003. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP-kinase cascades. *J Biol Chem* 278 (24):21989-97.

Shieh CC, Coghlan M, Sullivan JP, Gopalakrishnan M. 2000. Potassium channels: molecular defects, diseases, and therapeutic opportunities. *Pharmacol Rev.* 52(4):557-94.

Shinzawa K, Tsujimoto Y. 2003. PLA2 activity is required for nuclear shrinkage in caspase-independent cell death. *J Cell Biol.* 163(6):1219-30.

Simpson P, Savion S. 1982. Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. *Circ Res.* 50(1):101-16.

Skelton KH, Owens MJ, Nemeroff CB. 2000. The neurobiology of urocortin. *Regul Pept.* 93(1-3):85-92.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee KF. 1998. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* 20(6):1093-102.

Smith MD, Melton LA, Ensor EA, Packham G, Anderson P, Kinloch RA, Latchman DS. 2001. Brn-3a activates the expression of Bcl-x(L) and

promotes neuronal survival in vivo as well as in vitro. *Mol Cell Neurosci.* 17(3):460-70.

Spence J, Sadis S, Haas AL, Finley D. 1995. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol.* 15 (3): 1265-73.

Stephanou A, Brar B, Liao Z, Scarabelli T, Knight RA, Latchman DS. 2001. Distinct initiator caspases are required for the induction of apoptosis in cardiac myocytes during ischaemia versus reperfusion injury. *Cell Death Differ.* 8(4):434-5.

Sugden PH, Bogoyevitch MA. 1995. Intracellular signalling through protein kinases in the heart. *Cardiovasc Res.* 30(4):478-92.

Sun Y. 1999. Alterations of SAG mRNA in human cancer cell lines: requirement for the RING finger domain for apoptosis protection. *Carcinogenesis* 20(10):1899-903.

Sun Y, Tan M, Duan H, Swaroop M. 2001. SAG/ROC/Rbx/Hrt, a zinc RING finger gene family: molecular cloning, biochemical properties, and biological functions. *Antioxid Redox Signal.* 3(4):635-50.

Suzaki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, Tamaki T. 2002. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. *J Biol Chem.* 277(11):9614-21.

Swaroop M, Bian J, Aviram M, Duan H, Bisgaier CL, Loo JA, Sun Y. 1999. Expression, purification, and biochemical characterization of SAG, a ring finger redox-sensitive protein. *Free Radic. Biol. Med.* 27(1-2):193-202.

Swaroop M, Gosink M, Sun Y. 2001. SAG/ROC2/Rbx2/Hrt2, a component of SCF E3 ubiquitin ligase: genomic structure, a splicing variant, and two family pseudogenes. *DNA Cell Biol.* 20(7):425-34.

Takashi E, Ashraf M. 2000. Pathologic assessment of myocardial cell necrosis and apoptosis after ischemia and reperfusion with molecular and morphological markers. *J Mol Cell Cardiol.* 32(2):209-24.

Tanaka K, Suzuki T, Hattori N, MizunoY. 2004. Ubiquitin, proteasome and parkin. *BBA.* 1695 (1-3): 235-47.

Tanonaka K, Taguchi T, Koshimizu M, Ando T, Morinaka T, Yogo T, Konishi F, Takeo S. 1999. Role of an ATP-sensitive potassium channel

opener, YM934, in mitochondrial energy production in ischemic/reperfused heart. *J Pharmacol Exp Ther.* 291(2):710-6.

Tazi A, Dantzer R, Le Moal M, Rivier J, Vale W, Koob GF. 1987. Corticotropin-releasing factor antagonist blocks stress-induced fighting in rats. *Regul Pept.* 18(1):37-42.

Terui K, Higashiyama A, Horiba N, Furukawa KI, Motomura S, Suda T. 2001. Coronary vasodilation and positive inotropism by urocortin in the isolated rat heart. *J Endocrinol.* 169(1):177-83.

Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19(1):94-102.

Thuerauf DJ, Glembotski CC. 1997. Differential effects of protein kinase C, Ras, and Raf-1 kinase on the induction of the cardiac B-type natriuretic peptide gene through a critical promoter-proximal M-CAT element. *J Biol Chem.* 272 (11):7464-72

Tojo K, Sato S, Tokudome G, Ohta M, Kawaguchi Y, Sakai O, Nakagawa O, Nakao, K. 1996. Stimulation by corticotropin-releasing factor of atrial natriuretic peptide and brain natriuretic peptide secretions from cultured neonatal rat cardiomyocytes. *Biochem Biophys Res Commun* 225 (2): 340-6.

Toker A. 1998. Signaling through protein kinase C. *Front Biosci.* 3:D1134-D1147.

Tomei LD, Umansky SR. 2001. Apoptosis and the heart: a brief review. *Ann NY Acad Sci.* 946:160-8.

Tyers M, Jorgensen P. 2000. Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev.* 10(1):54-64.

Varshavsky A. 1996. The N-end rule: Functions, mysteries, uses. *Proc Natl Acad Sci USA.* 93 (22): 12142-9.

Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, . 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378(6554):287-92.

Vesterqvist O, Sargent CA, Grover GJ, Ogletree ML. 1996. Myocardial calcium-independent phospholipase A2 activity during global ischemia in isolated rabbit hearts. *Cardiovasc Res.* 31(6):932-40.

von Harsdorf R, Li PF, Dietz R. 1999a. Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. *Circulation* 99(22):2934-41.

von Harsdorf R, Hauck L, Mehrhof F, Wegenka U, Cardoso MC, Dietz R. 1999b. E2F-1 overexpression in cardiomyocytes induces downregulation of p21CIP1 and p27KIP1 and release of active cyclin-dependent kinases in the presence of insulin-like growth factor I. *Circ Res.* 85(2):128-36.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC. 1999. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 284(5412):339-43.

Weissman AM. 2001. Themes and variations on ubiquitylation. *Nature Rev Mol Cell Biol.* 2(3):169-78.

Weldon CB, Scandurro AB, Rolfe KW, Clayton JL, Elliott S, Butler NN, Melnik LI, Alam J, McLachlan JA, Jaffe BM, Beckman BS, Burow ME. 2002. Identification of mitogen-activated protein kinase kinase as a chemoresistant pathway in MCF-7 cells by using gene expression microarray. *Surgery* 132(2):293-301.

Wetzker R, Bohmer FD. 2003. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol.* 4 (8):651-7.

Whelan RD, Parker PJ. 1998. Loss of protein kinase C function induces an apoptotic response. *Oncogene* 16(15):1939-44.

Xu M, Wang Y, Ayub A, Ashraf M. 2001. Mitochondrial K(ATP) channel activation reduces anoxic injury by restoring mitochondrial membrane potential. *Am J Physiol Heart Circ Physiol.* 281(3):H1295-H1303.

Xu T, Jiao JH, Pence RA, Baertschi AJ. 1996. ATP-sensitive potassium channels regulate stimulated ANF secretion in isolated rat heart. *Am J Physiol.* 271(6 Pt 2):H2339-H2345.

Yamashita K, Kajstura J, Discher DJ, Wasserlauf BJ, Bishopric NH, Anversa P, Webster KA. 2001. Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1. *Circ Res.* 88(6):609-14.

Yang GY, Pang L, Ge HL, Tan M, Ye W, Liu XH, Huang FP, Wu DC, Che XM, Song Y, Wen R, Sun Y. 2001. Attenuation of ischemia-induced mouse brain injury by SAG, a redox-inducible antioxidant protein. *J Cereb Blood Flow Metab.* 21(6):722-33.

Yao Z, Tong J, Tan X, Li C, Shao Z, Kim WC, vanden Hoek TL, Becker LB, Head CA, Schumacker PT. 1999. Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes. *Am J Physiol.* 277(6 Pt 2):H2504-H2509.

Yaoita H, Ogawa K, Maehara K, Maruyama Y. 2000. Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction. *Cardiovasc Res.* 45(3):630-41.

Yeh ET, Gong L, Kamitani T. 2000. Ubiquitin-like proteins: new wines in new bottles. *Gene* 248(1-2):1-14.

Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH. 2000. Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res.* 86(6):692-9.

Zhao M, Brunk UT, Eaton JW. 2001a. Delayed oxidant-induced cell death involves activation of phospholipase A2. *FEBS Lett.* 509(3):399-404.

Zhao M, Eaton JW, Brunk UT. 2001b. Bcl-2 phosphorylation is required for inhibition of oxidative stress-induced lysosomal leak and ensuing apoptosis. *FEBS Lett.* 509(3):405-12.

Zhao ZQ, Nakamura M, Wang NP, Velez DA, Hewan-Lowe KO, Guyton RA, Vinten-Johansen J. 2000. Dynamic progression of contractile and endothelial dysfunction and infarct extension in the late phase of reperfusion. *J Surg Res.* 94(2):133-44.

Zhao ZQ, Vinten-Johansen J. 2002. Myocardial apoptosis and ischemic preconditioning. *Cardiovasc Res.* 55(3):438-55.

Zhu W, Zou Y, Aikawa R, Harada K, Kudoh S, Uozumi H, Hayashi D, Gu Y, Yamazaki T, Nagai R, Yazaki Y, Komuro I. 1999. MAPK superfamily plays an important role in daunomycin-induced apoptosis of cardiac myocytes. *Circulation* 100(20):2100-7.